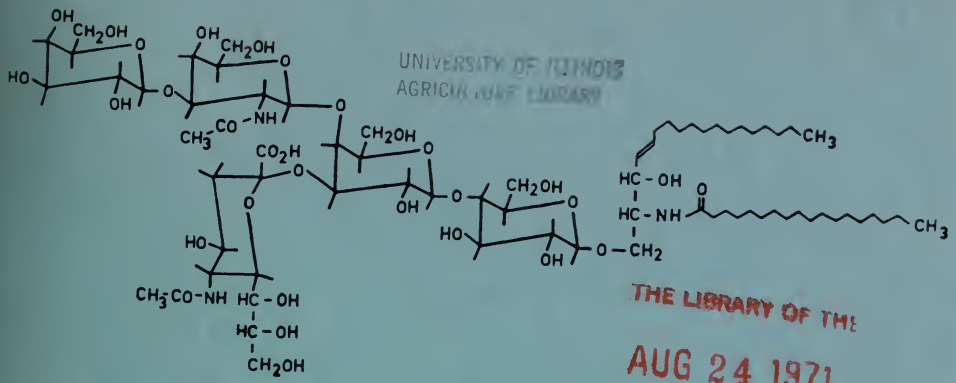
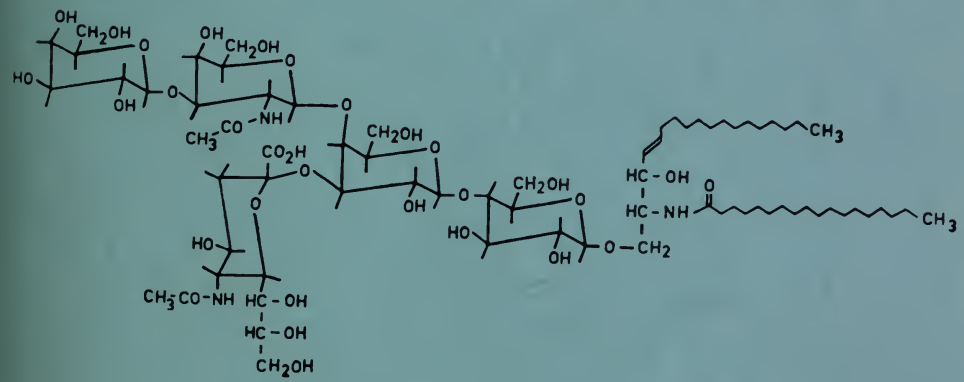
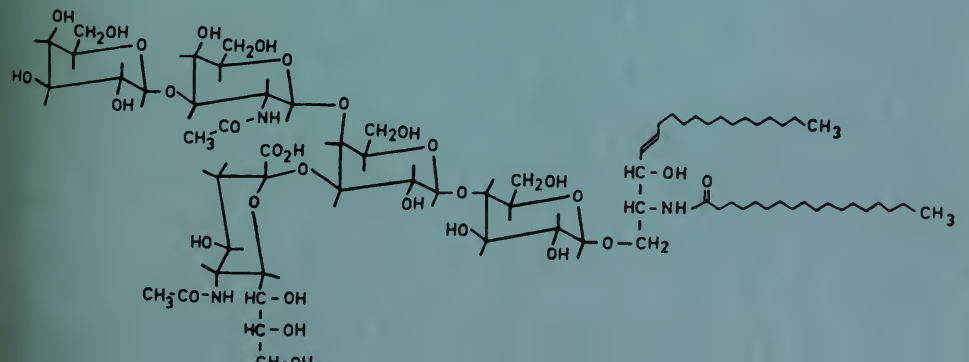


basic lipid methodology

by Patricia V. Johnston





basic lipid methodology

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January, 1971
College of Agriculture
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INTRODUCTION

THE SIZE AND DIVERSE NATURE of the group of compounds known as the lipids preclude the coverage of their chemistry to any extent in elementary courses in organic chemistry; this vast group is, in general, considered to be a field of study in itself. This publication has been prepared as a laboratory handbook for those with little previous experience in lipid analysis. Therefore, while a knowledge of basic organic chemistry is assumed, previous knowledge of lipid chemistry is not. The first chapter is an introduction to lipid chemistry. It includes definitions, classification, and basic structures of lipids. The rest of the book deals with the preparation and care of samples for lipid analysis and procedures for the analysis of commonly occurring lipids. A knowledge of newer techniques basic to the study of lipids is not assumed; therefore, the book also serves as an introduction to column, thin-layer, and gas-liquid chromatography.

Numerous references to original research papers are not given since this work is not intended to be an exhaustive account of all the methods available for the analysis of lipids. Rather, it is a personal account of methods tried, tested, and known to work. A few references to research papers are included where pertinent, and a selected bibliography is designed to lead the interested reader to a wider range of methods and applications. Analyses of only the more commonly encountered lipids are described, but the Suggested Further Readings includes some works dealing with the analysis of the less common lipid subclasses.

PATRICIA V. JOHNSTON *is Research Assistant Professor in the Children's Research Center and Assistant Professor of Food Science. She is also a member of the Nutritional Sciences Faculty. Developmental neurochemistry and neurobiology are Dr. Johnston's main research interests. She is especially interested in pre- and postnatal conditions, such as malnutrition and drug therapy, which may affect the development of the lipid-rich myelin sheath of nerve cells.*

This book was written in part while Dr. Johnston was supported by funds from the Hatch NC74 committee.

The figure on the cover is a monosialoganglioside.

TABLE OF CONTENTS

I. Lipids: Definitions, Classification, Nomenclature

THE TERM LIPID describes a large group of compounds of a chemically diverse nature. This book employs a simple definition and classification system, as no system has been universally accepted. Lipids may be defined as compounds found in living organisms and generally insoluble in water but soluble in organic solvents. There are exceptions to this definition since some lipids are sparingly soluble in water while others are soluble in a very limited number of organic solvents. In general, however, this definition holds.

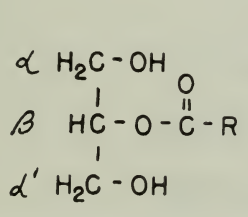
Three major lipid classes exist, namely the simple lipids and the two general groups of polar lipids, the glycerophosphatides and the sphingolipids. We shall consider each of these classes individually. Examples of the structural formulae of the lipids mentioned are shown.

SIMPLE LIPIDS

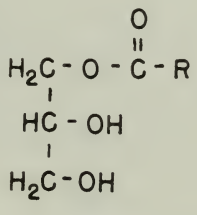
These compounds comprise the neutral lipids such as the glycerides, esters of fatty alcohols, and lipids derived from these compounds by alkaline or acid hydrolysis.

Neutral Glycerides

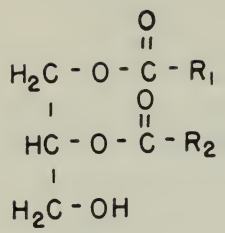
The neutral glycerides are fatty acid esters of glycerol, and although the most abundant are the triglycerides, mono- and diglycerides do occur naturally. The glycerides are named according to the position of the fatty acid substituent on the glycerol moiety; thus two isomers of mono- and diglycerides exist. In a triglyceride all the glyceride hydroxyl groups are esterified.



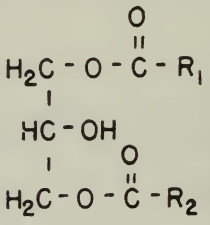
monoglyceride, β-form



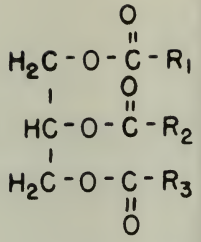
monoglyceride, α-form



diglyceride, α,β-form

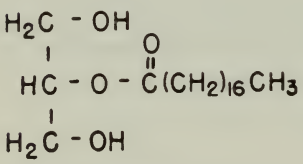


diglyceride, α,α'-form

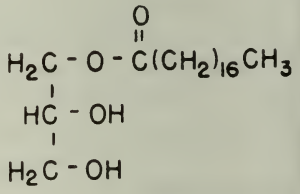


triglyceride

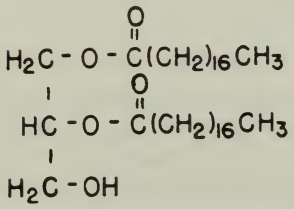
The carbons of the glycerol moiety are often designated 1, 2, and 3 instead of α, β, and α'. R_1, R_2, and R_3 refer to the alkyl chain of the esterifying fatty acids. In general, glycerides are named by the trivial name for the fatty acids. For example, if stearic acid, $CH_3(CH_2)_{16}$-COOH, is the esterifying fatty acid, we can have:



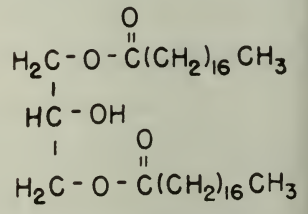
2 (or β)-monostearin



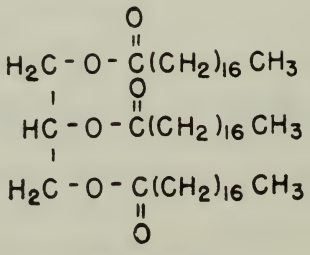
1 (or α)-monostearin



1,2 (or α,β)-distearin

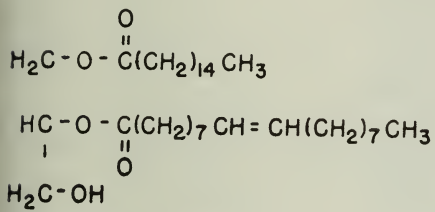


1,3 (or α,α')-distearin

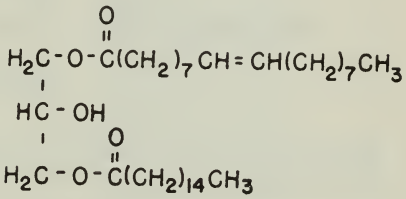


tristearin

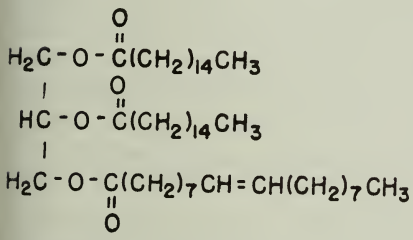
When the fatty acid composition is mixed, the position of each fatty acid is specified when known. Thus, introducing oleic $CH_3(CH_2)_7$-$CH = CH(CH_2)_7COOH$ and palmitic $CH_3(CH_2)_{14}$ $COOH$ acids, we can have as examples:



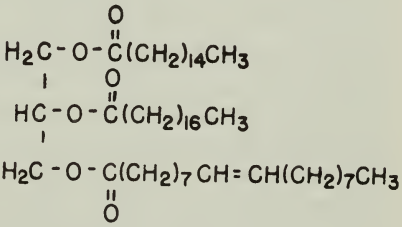
1-palmitoyl-2-olein



1-oleoyl-3-palmitin



1,2-dipalmitoyl olein



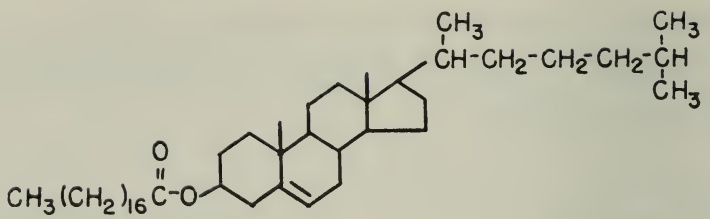
1-palmitoyl-2-stearoyl-3-olein, or (since the 1 and 3 positions are equivalent) 1-oleoyl-2-stearoyl-3-palmitin

When the fatty acids are more complicated and not generally known by any trivial name, they must of course be named systematically. The generally accepted nomenclature for fatty acids is described under Derived Lipids (p. 4).

Esters of Fatty Alcohols

Alcohols that are relatively insoluble in water but are soluble in organic solvents fall under the class of fatty (or lipid) alcohols. This class, therefore, includes long-chain aliphatic alcohols (more than 8 carbons), aromatic alcohols such as cholesterol, and other steroids, including the vitamin D group. Vitamins A and E are also lipid alcohols, but because of the vast literature devoted to the vitamins we shall not discuss them further here. Cholesterol is the alcohol with which we shall be most concerned. Cholesterol exists in nature in the esterified state and in the free form. The stearic acid ester of cholesterol is shown (p. 4).

The esters of long-chain alcohols and fatty acids are known as waxes; natural waxes such as beeswax and wool waxes are mixtures of



cholesterol ester

these esters. Trace amounts of other components, such as hydrocarbons, are also found in natural waxes.

Derived Lipids

If the products obtained on hydrolysis of a lipid are soluble in organic solvents and relatively insoluble in water, they are termed derived lipids. From our point of view, the most important are the fatty acids. We shall now discuss their structure and nomenclature in some detail. For the most part, the fatty acids in mammalian tissues and body fluids are straight chain and contain an even number of carbon atoms. Hydroxy, keto, and branch chain fatty acids also occur; indeed, the nervous system of mammals contains considerable amounts of 2(α)-hydroxylated fatty acids. Most fatty acids found in tissues are in ester or amide linkages, but small amounts of free fatty acids (FFA) do occur and are of great importance metabolically both in supplying energy and in lipid biosynthesis.

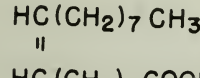
It is usual to divide the fatty acids into two main classes, saturated and unsaturated. Since aliphatic acids are regarded as derivatives of the hydrocarbons which have the same number of carbon atoms, the names of fatty acids are derived from the appropriate parent hydrocarbon. Saturated fatty acids are named, according to the modified Geneva system, by replacing the terminal "e" of the parent hydrocarbon name with the suffix "oic." Thus, the saturated fatty acid that is related to the hydrocarbon octane, $CH_3(CH_2)_6CH_3$, is known as octanoic acid. Most fatty acids, however, were known and named before the Geneva convention, and the use of their non-systematic ("trivial") names persists. The accompanying tables of fatty acids, therefore, include the trivial names as well as the systematic names. Some commonly occurring saturated fatty acids are shown in Table 1.

The simplest unsaturated fatty acids have the empirical formula $C_nH_{2n-2}O_2$; that is, they contain only one double bond. They are named by replacing the "e" of the corresponding unsaturated hydrocarbon with the suffix "oic"; thus one has octenoic acid, decenoic acid, and so on. These fatty acids are termed "monoenoic." Fatty acids with two, three, four, five, and more double bonds are named by taking the stem of the

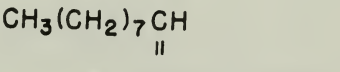
Table 1. — Some Saturated Fatty Acids

Systematic name	Trivial name	Formula
Butanoic	Butyric	$CH_3(CH_2)_2COOH$
Hexanoic	Caproic	$CH_3(CH_2)_4COOH$
Octanoic	Caprylic	$CH_3(CH_2)_6COOH$
Decanoic	Capric	$CH_3(CH_2)_8COOH$
Dodecanoic	Lauric	$CH_3(CH_2)_{10}COOH$
Tetradecanoic	Myristic	$CH_3(CH_2)_{12}COOH$
Hexadecanoic	Palmitic	$CH_3(CH_2)_{14}COOH$
Octadecanoic	Stearic	$CH_3(CH_2)_{16}COOH$
Eicosanoic	Arachidic	$CH_3(CH_2)_{18}COOH$
Docosanoic	Behenic	$CH_3(CH_2)_{20}COOH$
Tetracosanoic	Lignoceric	$CH_3(CH_2)_{22}COOH$

name of corresponding hydrocarbon, octa-, deca-, etc., and adding the appropriate ending: "-dienoic" (2 double bonds), "-trienoic" (3 double bonds), etc. Fatty acids with multiple double bonds are referred to collectively as polyunsaturated fatty acids (PUFA) and individually as dienoic, trienoic, tetraenoic, pentaenoic, and hexaenoic fatty acids (see Table 2). A name must also, of course, designate both the position of the double bonds along the chain and their geometric configuration: *cis* or *trans*. Double bonds are assumed to be *cis*, unless a statement is made to the contrary. This convention has been adopted because most naturally occurring fatty acids have their double bonds in the *cis* configuration; moreover, most common *trans* isomers have a different trivial name which is used, so the problem is often completely circumvented. For example, octadecenoic acid with a double bond on the 9th carbon has both *cis* and *trans* forms. The *cis* form has the trivial name of oleic acid, and the *trans* form is known as elaidic acid. Similarly, when



cis form: oleic acid



trans form: elaidic acid

both double bonds of octadecadienoic acid are *trans*, the acid is termed linolelaidic acid. While *trans* fatty acids are rare as natural constituents, they do occur naturally in some plants and animals; moreover, when included in the diet, they are deposited in body tissues (*1*). They are, therefore, nutritionally significant. A further discussion of *trans* fatty acids is included in the description of their analysis (see p. 94).

While the naming of geometric isomers is relatively straightforward, the designation of double bond position is confused by the fact that

Table 2. — Some Unsaturated Fatty Acids

Systematic name	Trivial name	Formula
cis-9-hexadecenoic	Palmitoleic	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
cis-9-octadecenoic	Oleic	$CH_3(CH_2)_7\overset{cis}{CH=CH}(CH_2)_7COOH$ } see text
trans-9-octadecenoic	Elaidic	$CH_3(CH_2)_7\overset{trans}{CH=CH}(CH_2)_7COOH$ } see text
cis-9,12-octadecadienoic	Linoleic	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$
cis-6,9,12-octadecatrienoic	γ-Linolenic	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_4COOH$
cis-9,12,15-octadecatrienoic	Linolenic	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$
cis-5,8,11-eicosatrienoic	.....	$CH_3(CH_2)_7CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$
cis-5,8,11,14-eicosatetraenoic	Arachidonic	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$

several conventions exist. To be able to follow the literature, one must know all the conventions. In the simplest procedure the terminal carboxyl group carbon is number 1 and the rest of the chain is 2, 3, 4, etc. Thus palmitoleic acid named systematically becomes 9-hexadecenoic acid, $CH_3(CH_2)_5\overset{9}{C}H = \overset{1}{C}H(CH_2)_7COOH$. However, especially in older literature, lengthier conventions are used. The Greek letter Δ is used to indicate the presence of double bonds, and both carbon atoms participating in the bonds are indicated. Palmitoleic acid then becomes $\Delta^{9,10}$-hexadecenoic acid. Δ^9-hexadecenoic acid is also used.

There is a growing tendency to use a shorthand designation for fatty acids. This is very useful especially when long lists of fatty acids are given as, for example, in the complete gas chromatographic analysis of the fatty acids of natural lipids. In this convention the Greek letter ω is used to refer to the terminal carbon atom away from the carboxyl group. The position of the double bond is then indicated with respect to the ω carbon. Palmitoleic acid is, therefore, the ω 6-hexadecenoic acid. The length of the carbon chain and the number of double bonds is indicated by the shorthand form C16:1, where the number before the colon gives the chain length and the number after the colon indicates the number of double bonds. Palmitoleic acid is, therefore, designated C16:1 ω 6.

When there is more than one double bond a question arises; namely, is it sufficient to define the position of only one double bond with respect to the ω carbon atom? To answer this, we must consider still another way of classifying fatty acids. Polyenoic acids may be classified as *conjugated* or *unconjugated* (*nonconjugated* is also used) depending on the relative position of the double bonds. If the double bonds are separated by one or more single-bonded carbon atoms, $-C=C-C_n-C=C-$, the acid is said to be *unconjugated*. When double-bonded carbon atoms are adjacent to each other, $-C=C-C=C-$, the acid is termed *conjugated*. Fatty acids from mammalian sources are usually *unconjugated*. Double bonds are usually separated by one single-bonded carbon atom; that is, by a single methylene ($-CH_2-$) group. It is, therefore, perfectly correct in such cases to define double bond position by reference to the first double bond from the ω carbon atom. Thus, the commonly occurring mammalian fatty acids, 5, 8, 11-eicosatrienoic acid and 5, 8, 11, 14-eicosatetraenoic (arachidonic) acid, can be referred to as 20:3 ω 6 and 20:4 ω 6 respectively. If, however, the double bonds are conjugated, then reference must be made to this fact. If the acid is unconjugated but double bonds are separated by more than one single-bonded carbon atom, then clearly one of the lengthier descriptions of the structure must be used.

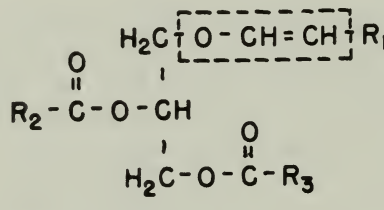
The fatty acids listed in Tables 1 and 2 occur widely in nature, as do numerous others. Linoleic acid is of great importance to mammals: it

must be included in their diets to prevent a deficiency syndrome since they cannot synthesize it. For this reason linoleic acid is called an essential fatty acid (EFA). Arachidonic and γ-linolenic acids, synthesized in mammals from linoleic acid, are also sometimes termed essential fatty acids since they protect and cure the EFA deficiency syndrome.

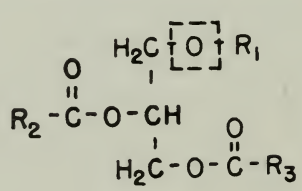
Hydroxy, keto, branched chain, and cyclic fatty acids all exist. Examples of such acids and a source of each are shown in Table 3. The only hydroxy fatty acids of importance in mammals are the 2(α)-hydroxy substituted fatty acids found in cerebrosides and sulfatides (see p. 15). Some hydroxy and branched chain acids may be ingested by man in oils from plant sources (such as ricinoleic acid in castor oil), and small quantities of hydroxy and keto acids may also be present in man's diet due to oxidative breakdown of fats (*2, 3*).

Glyceryl Ethers

Neutral lipids with ether or vinyl ether linkages have been isolated and characterized. In these lipids an ester-linked fatty acid of a glyceride is replaced by a vinyl ether (α,β unsaturated) linkage or an ether linkage. Thus, one may have a *cis*-1-(alkenyl) ether of a 2,3-diacyl glycerol with the general formula:



and glycerol ethers with the formula:



These lipids are of importance in fish and many invertebrates (*4*).

PHOSPHOGLYCERIDES

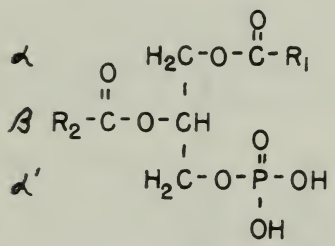
Phosphoglycerides are defined as lipids which, on hydrolysis, produce derived lipids plus inorganic phosphate and glycerol.

Table 3. — Hydroxy, Keto, Branched Chain, and Cyclic Fatty Acids

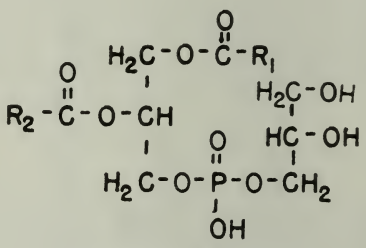
Systematic name	Trivial name	Formula	Source
2-hydroxytetracosanoic	Cerebronic	$CH_3(CH_2)_{21}CH(OH)COOH$	Brain lipids
12-hydroxy-9-octadecenoic	Ricinoleic	$CH_3(CH_2)_5CH(OH)CH_2CH=CH(CH_2)_7COOH$	Castor oil
4-keto-9,11,13-octadecatrienoic	Licanic	$CH_3(CH_2)_3CH=CHCH=CHCH=CH(CH_2)_4C(=O)(CH_2)_2COOH$	Seeds of *Coupeia grandiflora*
3-methylbutanoic	Isovaleric	$CH_3CH(CH_3)CH_2COOH$	Dolphin and porpoise oils
13-(2-cyclopentenyl)tridecanoic	Chaulmoogric	$CH_2-CH_2-CH(CH_2)_{12}COOH$ (with $CH=CH$ in ring)	Chaulmoogra oil

Phosphatidic Acids, Phosphatidyl Glycerols, and Polyglycerophosphatides

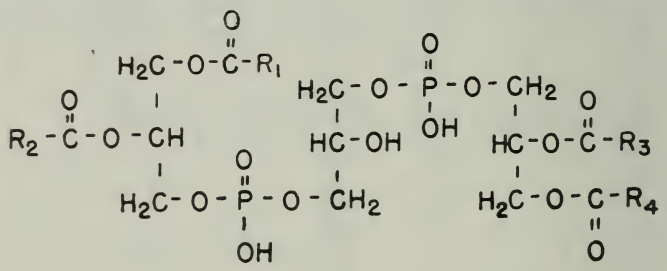
The simplest phosphoglycerides are the phosphatidic acids. In these lipids two of the glycerol —OH groups are esterfied with fatty acyl groups and the third with phosphoric acid (see also phosphonolipids, p. 13). Closely related to the phosphatidic acids are the phosphatidyl glycerols and the polyglycerophosphatides. Cardiolipin, a polyglycerophosphatide, occurs mainly as a mitochondrial lipid.



phosphatidic acid



phosphatidyl glycerol



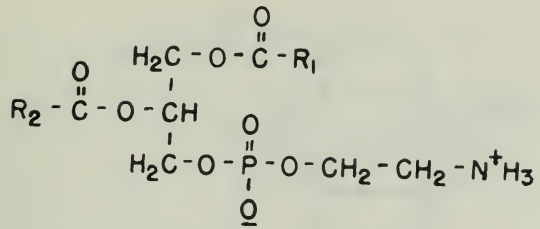
cardiolipin

Phosphatidyl Ethanolamines, Cholines, Serines, and Inositols

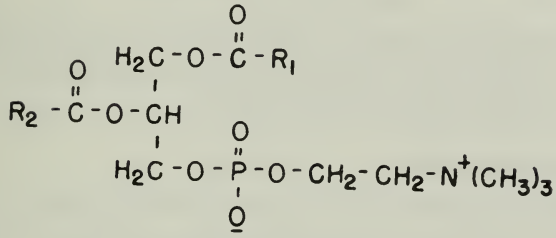
This subclass contains some of the most abundantly occurring phospholipids. In these compounds the phosphoric acid group of the parent compound, a phosphatidic acid, is linked to either ethanolamine, choline, serine, or myoinositol. At least three subclasses of phosphoinositide exist, differing in the number of phosphoric acid residues they contain.

Phosphatidyl choline is known by the generally accepted trivial name of lecithin. Phosphatidyl ethanolamine, however, should not be termed "cephalin," as this trivial name originally referred to a mixture and has since lost its meaning.

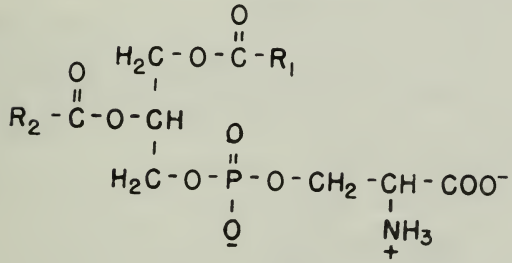
It will be noted that the phosphoglycerides are amphiphilic compounds; that is, one end of the molecule consists of a hydrophobic region of long-chain alkyl groups while the other end is hydrophilic, consisting of the ionic phosphoryl group and its esterifying molecule. Note also that the β carbon of the glycerol moiety is asymmetrical. Phosphoglyc-



phosphatidyl ethanolamine



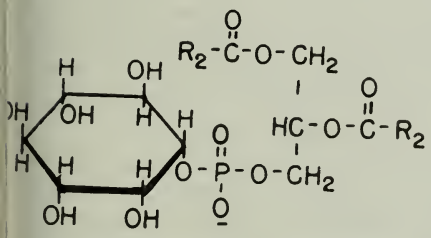
phosphatidyl choline



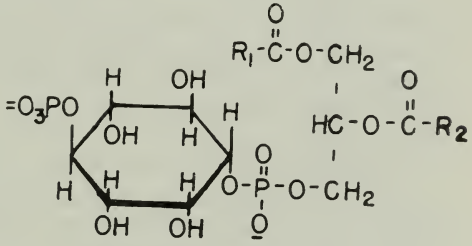
phosphatidyl serine

erides have been found to have the same stereochemical configuration as L-α-glycerophosphate. They are, therefore, more correctly termed L-α-phosphatidyl enthanolamine, and so on. In general, unsaturated fatty acids esterify the β position in all phosphoglycerides and saturated fatty acids esterify the α position.

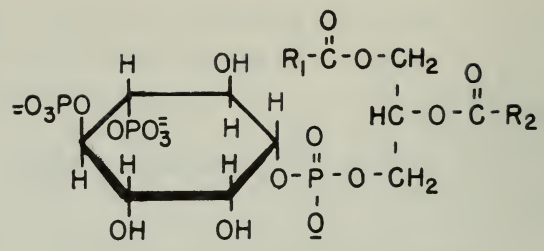
The three commonly occurring phosphoinositides are shown below and on page 12. The monophosphorus compound, phosphatidyl inositol,



phosphatidyl inositol



diphosphoinositide



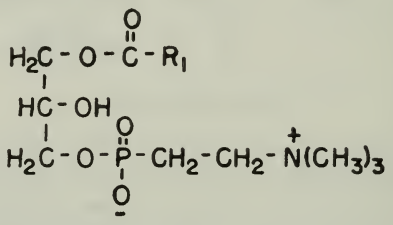
triphosphoinositide

occurs in skeletal and cardiac muscle; the di- and triphosphoinositides constitute a considerable proportion of the phospholipids in brain and occur only as trace amounts in other tissues.

Lysophosphoglycerides

In lysoglycerophosphatides only one alcoholic group is esterified by a fatty acid; the other one is free. The only common naturally occurring member of this subclass is lysophosphatidyl choline (lysolecithin), which usually has the β-OH group free.

Lysolecithin occurs to an appreciable extent in blood serum. Between 6 and 10 percent of the total lipid phosphorus in human blood serum is lysolecithin (*5*). There is evidence for the occurrence of the monoacyl compounds of all the other phosphoglycerides and for isomeric forms in which the α-OH group is free.

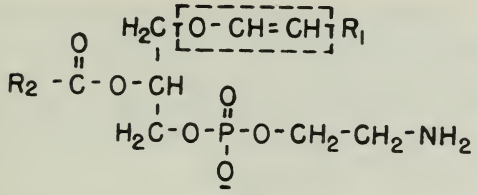


lysolecithin

Plasmalogens

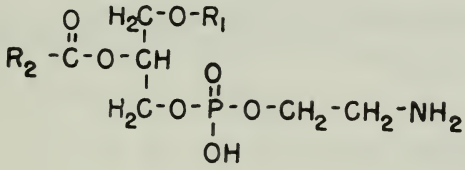
Plasmalogens are monovinyl-ether, monofatty-acyl phosphoglycerides. They are known generally by the trivial name plasmalogens and occur in a wide range of tissues in most species. Typically, the fatty acyl group at the α position is replaced by an alkenyl group so that in place of the ester linkage there is a vinyl ether linkage. The most abundant plasmalogen in a tissue is usually the ethanolamine derivative.

Plasmalogens are sometimes named by replacing the terminal "yl" of the phosphatidyl analog by "al," so that we have phosphatidal ethanolamine, phosphatidal choline, etc. This method, however, is subject to error due to the close similarity in the names and has not, therefore, found universal acceptance. It is preferable when referring to plasma-



ethanolamine plasmalogen

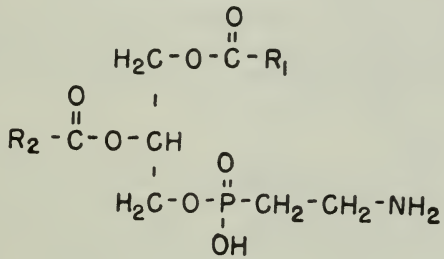
logens to use either "ethanolamine plasmalogen" or "vinyl ether phosphatidyl ethanolamine." These terms clearly distinguish the plasmalogens from the diacyl phosphoglyceride. Still another type of phosphoglyceride occurs. This is the relatively rare alkoxy glycerophosphatide in which the α ester linkage is replaced by an ether linkage.



alkoxy phosphatidyl ethanolamine

Phosphonolipids

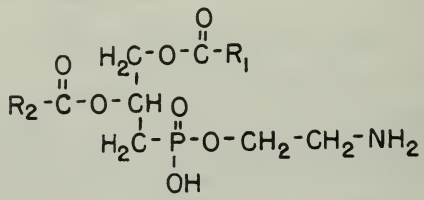
Some years ago an entirely new group of phosphorus-containing lipids was discovered by two groups of investigators, one studying the lipids of the sea anemone, *Anthopleura elegantissima* (*6*), the other studying the proteolipid fraction of ciliate protozoa of sheep rumen (*7*). This group of lipids contains phosphonic acid in place of phosphoric acid. The first clues to the existence of these lipids came with the isolation of 2-aminoethylphosphonic acid $(OH)_2-\overset{\overset{O}{\|}}{P}-CH_2-CH_2-NH_2$, suggesting that this replaced 2-aminoethylphosphoric acid $(OH)_2-\overset{\overset{O}{\|}}{P}-O-CH_2-CH_2-NH_2$ in some lipids. A typical glycerol phosphonolipid would, therefore, have the structure:



dialkyl glyceryl-(2-aminoethyl)-phosphonate

Many types of phosphonolipids have now been isolated and characterized, including sphingolipid and plasmalogen classes.

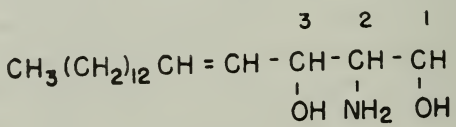
Phosphonolipids have also been synthesized; Baer and Stanacev (*8, 9*) have prepared glycerol phosphonolipids of the type on page 13 and of a type in which the glycerol is bound directly to the phosphorus by a carbon-phosphorus bond.



More recently Chacko and Hanahan (*10*) have synthesized the monoether phosphatidyl aminoethylphosphonate and have confirmed the presence of both mono- and diester phosphonolipids in *Tetrahymena pyriformis* (a ciliate).

SPHINGOLIPIDS

Sphingolipids are compounds which on hydrolysis yield sphingosine (or a closely related compound), derived lipids, and water soluble products. Unlike the complex lipids discussed so far, this group does not contain the glycerol carbon skeleton. Rather, the common link is sphingosine, an amino alcohol with the following structure:



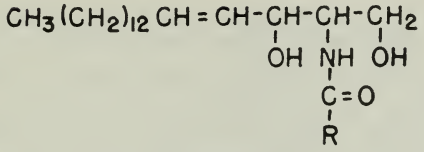
sphingosine

Sphingosine carbons are numbered starting with the primary hydroxyl group. The configuration of the molecule at C2 is D, and the relationship of C3 to C2 is *erythro*. The double bond has the *trans* configuration. The systematic name for sphingosine is, therefore, D-*erythro*-1,3-dihydroxy-2-amino-4-*trans*-octadecene.

Molecules closely related to sphingosine, and which replace it in some sphingolipids, include dihydrosphingosine (fully saturated sphingosine) and C20 homologues of sphingosine.

Ceramides

The simplest and most fundamental sphingolipids are called ceramides. In these compounds the amino group of sphingosine (or a sphingosine-related compound) is in an amide linkage with a fatty acid. Amide, ether, and vinyl ether linkages are stable to alkali while ester

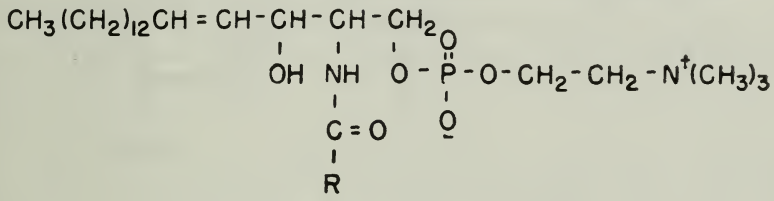


ceramide

linkages are readily broken by alkali. This difference is used to advantage in analytical procedures.

Sphingomyelin

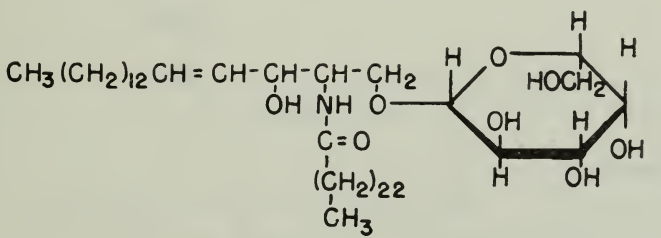
Sphingomyelin is a phosphorus- and choline-containing sphingolipid. The primary alcohol group of a ceramide is joined via an ester linkage to phosphoric acid which is in turn esterified with choline. Some 10 percent of the lipid phosphorus in brain is sphingomyelin; kidney, spleen, erythrocytes, and plasma are also rich in sphingomyelin.



sphingomyelin

Glycolipids: Cerebrosides and Sulfatides

In the general group of lipids called glycolipids, or glycosyl ceramides, the primary hydroxyl group of a ceramide is linked by a glycosidic bond to a monosaccharide or oligosaccharide chain. A cerebroside is a ceramide linked to a monosaccharide, usually galactose. Cerebrosides are important constituents of nervous tissue; they are major constituents of the myelin sheath. Nervous tissue cerebrosides are of two types: those containing normal fatty acids and those containing 2-hydroxy fatty acids. A typical cerebroside containing lignoceric acid is known by the trivial name "kerasin" (shown below). When the fatty

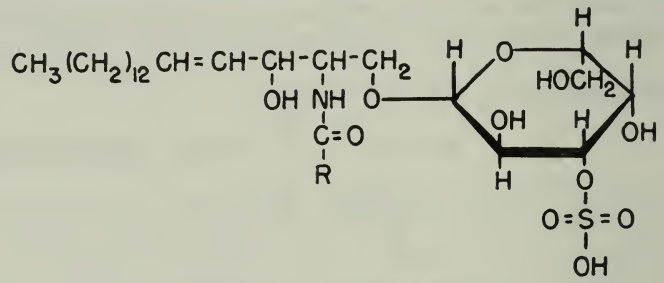


ceramide galactoside (kerasin)

acid constituent is 2-hydroxytetracosanoic (cerebronic) acid, the cerebroside is called "phrenosin." The cerebrosides containing 9-tetracosenoic (nervonic) and 2-hydroxy-9-tetracosenoic (oxynervonic) acids are known as "nervon" and "oxynervon" respectively.

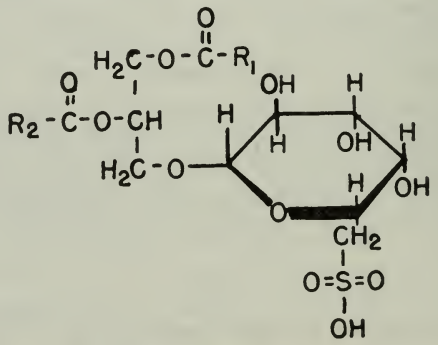
Glycolipids of other types are found in spleen, liver, plasma, and erythrocytes. These include ceramide-dihexosides, in which a ceramide is linked to a galactose disaccharide or a glucose-galactose disaccharide; ceramide-trihexosides; and ceramide-tetrahexosides.

Sulfatides are sulfate esters of cerebrosides in which the sulfate group is found on C3 of the galactose moiety. Like the cerebrosides, the sulfatides are abundant in nervous tissues. These sulfatides also occur with 2-hydroxy fatty acid constituents, but to a lesser extent than do nervous tissue cerebrosides. In other tissues, such as kidney, sulfatides of ceramide dihexosides have been found.



sulfatide (cerebroside sulfate)

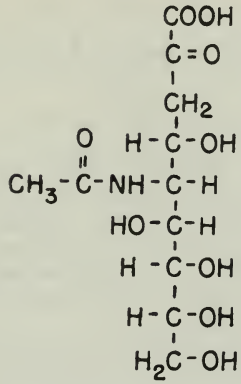
Sulfatides should not be confused with sulfolipids, a group of plant lipids containing sulfonic acid and having the general formula:



sulfolipid

Gangliosides

Gangliosides are a complex group of glycosphingolipids which differ from the other glycolipids in containing sialic acids. Sialic acids are

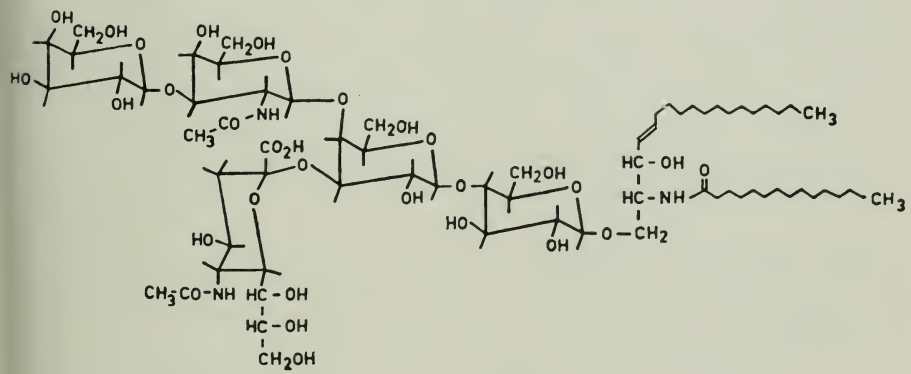


D (−)N-acetylneuraminic acid

N-acyl derivatives of neuraminic acid; the common constituent of gangliosides is N-acetylneuraminic acid.

High levels of gangliosides occur in nervous tissue; they are believed to be located specifically in the neurons. Other gangliosides, containing N-glycoylneuraminic acid, have been found in spleen and in red blood cell stroma.

The gangliosides of brain have been most extensively studied and have been separated into three main types — mono-, di-, and trisialogangliosides — according to their sialic acid content. The major monosialoganglioside has been shown to have the following structure:



A group of relatively recently discovered compounds, the prostaglandins, are also classified as lipids. The study of these substances is rapidly developing into a separate field, so they are not considered in this text. A review of the chemistry of the prostaglandins is included in the list of Suggested Further Readings (p. 99).

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II. Preparation and Handling of Samples for Analysis of Lipid Constituents

DURING THE EXTRACTION OF LIPIDS from tissues and their subsequent handling during analysis the lipid chemist must constantly battle his two worst enemies, oxidation and contamination. We cannot overstress the need to set up conditions in the laboratory that will prevent oxidation of the lipids and intrusion by contaminants (lipid and nonlipid). It is here that the inexperienced lipid analyst most frequently fails. Before discussing extraction procedures we shall consider some basic rules to follow when working with lipids.

GENERAL TECHNIQUES IN LIPID CHEMISTRY

Prevention of Oxidation

When exposed to air the unsaturated moieties of lipids rapidly oxidize, a process that is accelerated by light. It is absolutely essential that the lipids be manipulated in an oxygen-free environment. This is generally achieved by carrying out operations under an atmosphere of nitrogen. A lipid laboratory should have nitrogen supplies at every point where lipids are to be handled. This can be accomplished by running several branch lines of polyethylene tubing from a tank (or tanks) of nitrogen. The nitrogen flow can be regulated by constricting the tubing with clamps or by inserting Teflon stopcocks or flow regulators at suitable points. Glass stopcocks should not be used, as the grease from them may get blown down the lines and contaminate samples. Several examples of when and how to use nitrogen follow:

1. When filtering a solution containing lipids, attach a line from the nitrogen to the stem of a funnel and invert over the filtration apparatus, letting a gentle flow of nitrogen continue until filtration is complete.

2. When placing lipid samples in a dessicator to dry, first flush out the dessicator with nitrogen, evacuate, and repeat the process. When releasing the vacuum in the dessicator, do so by letting in nitrogen, not air. Any apparatus in which lipid is placed should be fitted with three-way stopcocks so that it can be flushed through with nitrogen.

3. Other manipulations of the lipid can be carried out under a plastic "tent" within which a slight positive pressure of nitrogen is maintained. Setups of this type can readily be made by purchasing plastic "glove bags," which are available from a number of supply houses.

4. When dealing with lipid dissolved in small volumes of solvent, it is often convenient to remove the solvent under a stream of nitrogen.

For this purpose a manifold (a tube having several outlets) attached to a nitrogen line is very useful. The nitrogen stream should be directed through disposable glass pipets, which can be discarded after use with one sample.

In general, lipid samples should be blanketed by nitrogen during all operations. This is not so necessary when the lipid is in an atmosphere saturated with solvent vapor. If lipid or tissues have to be stored for a while, they should be frozen rapidly and kept in their containers at −20°C (or below) in a plastic bag flushed out with nitrogen.

In addition to using a nitrogen atmosphere it is often convenient to add to the lipid an antioxidant such as 2,6-di-t-butyl-*p*-cresol, more commonly called butylated hydroxytoluene (BHT). The usual addition of BHT is 0.1 percent of the weight of the lipid.

Lipids should never be stored in chloroform-methanol solution as this leads to breakdown of phosphoglycerides.

Additional hazards of breakdown occur during removal of tissues from animals and during maceration of the sample. Here, lipolytic enzymes may be activated and break down some of the lipids. It is, therefore, essential that dissection and maceration are carried out as rapidly as possible.

Elimination of Possible Contaminants

Lipids and compounds which mimic lipids in their chromatographic behavior are likely to enter samples in a number of ways. The only way to avoid artifacts and contaminants is to ensure that all laboratory personnel abide by a set of rigid rules. The following list of suggested rules illustrates the vast number of sources of contamination.

1. All reagent grade solvents should be redistilled to remove non-volatile impurities. Distillation should be carried out in glass apparatus and solvents stored in glass containers. Chloroform should be stabilized by adding 0.25 percent (by vol.) methanol and stored in a dark bottle.

2. Corks, rubber stoppers, plastic film wraps, etc., should not be used to close containers containing lipid samples, especially when these materials may come in contact with solvent vapors. Rubber tubing should not be used. Contact of solvent with any tubing should be avoided.

3. The use of grease on stopcocks and vacuum systems should be avoided. Teflon stopcocks are the best solution. Good ground glass apparatus on rotary vacuum evaporators and similar equipment will usually produce good leakless seals without the use of grease.

4. Personnel should be trained never to put their fingers inside vessels to be used for lipid work. Fingerprints are a rich source of lipids.

5. Smoking near chromatographic equipment should be forbidden as tobacco smoke is a rich source of chromatographic artifacts.

6. Vapors from vacuum pumps and other apparatus should be vented into a hood.

7. If at all possible, glassware should be washed in chromic acid and rinsed thoroughly in deionized water.

8. Finally, it is good practice in the lipid laboratory to run blanks of procedures to check for possible artifacts and contaminants.

Rouser *et al.* (*1*) have demonstrated the presence of an impressive number of potential contaminants found in a lipid laboratory.

Unwanted Emulsions and Other Hazards in Lipid Chemistry

The beginner in lipid chemistry encounters hazards in addition to those of oxidation and contamination. Invariably he will experience the formation of unwanted and seemingly intractable emulsions. As we shall see later, procedures in lipid analysis frequently call for the partitioning of lipids and nonlipids between aqueous and organic solvent phases. The very property of lipids which makes them important in the assembly of biological membranes, namely their amphiphilic nature, means that they are frequently good emulsifiers. As a consequence, the beginner finds that phases refuse to separate; often there is a band of emulsified material where the interface should be, or the whole system becomes emulsified. Emulsions are easier to prevent than to cure. Prevention is therefore emphasized.

Whenever a procedure calls for the intermixing of aqueous and nonaqueous phases, this should be achieved by swirling the contents of the container (usually a separatory funnel) and inverting the vessel several times. Vigorous shaking, especially when phospholipids are present, will form emulsions. Emulsions also readily form when soaps are present, such as when a soap solution is being extracted with ether or hexane to remove non-saponifiable lipids. Generally, however, swirling and inverting the vessel rather than shaking it vigorously will prevent the formation of hard-to-break emulsions.

If emulsions are formed, there are several techniques for breaking them. Should the whole system form a very permanent-looking emulsion, centrifugation (at 600 to 700 $\times$ g) will break it most quickly. Other emulsion-breaking techniques are generally useless and a waste of time. If only part of the system is emulsified and the interface can be detected, the emulsion can often be dispersed by using a disposable pipet to add a little ethanol at the interface. Adding a salt, such as sodium sulfate, and gently swirling will also tend to break emulsions. Generally, however, centrifugation gives clean and quick results and is the preferred method.

Numerous other tedious difficulties and potential dangers (to personnel as well as to samples) are encountered in the lipid laboratory. In general these are discussed in the text as the situations arise. A common problem should, however, be mentioned immediately. Fre-

quently, a beginner complains that results are not reproducible. There are many possible reasons for this, but the adoption of one simple rule often solves the problem. Lipids must *never* be sampled from the solid or semi-solid state unless efforts have been made to homogenize the sample. Lipids extracted from natural materials are not homogeneous: they crystallize and solidify at different rates and different temperatures so that all parts of the sample are not necessarily the same. The sample can be homogenized by grinding it under nitrogen in a mortar, but it is more common and more effective to dissolve the lipids in a known volume of solvent and to take aliquots from this.

EXTRACTION OF LIPIDS FROM VARIOUS SOURCES

Samples from which lipids are to be extracted should be as fresh as possible. If tissues dissected from animals cannot be extracted immediately, they should be frozen by plunging them into liquid nitrogen. They should then be stored at −20°C or below in closed containers that have been flushed out with nitrogen. Blood samples should not be drawn unless it is certain that they can be treated immediately.

It is not possible to describe in detail an extraction procedure that is applicable to all types of material. In changing from one source to another, some modification of procedure is usually necessary. Some general remarks, however, can be made about maceration of samples.

If the sample is no larger than a few grams, it can be ground most efficiently with a tissue homogenizer of the Potter Elvehjem or Tenbroech type. These homogenizers consist of ground glass tubes containing closely fitting pestles. They can be used for grinding by hand, or the pestle can be turned mechanically by attaching it to a high speed motor. The tube should be placed in ice while grinding. Larger samples can be handled in Waring Blendors, or by using a mortar and pestle.

The choice of the macerating method depends to a great degree on the toughness of the sample. When dealing with tough tissues such as skin or samples rich in connective tissue, it is best to freeze the sample in a steel mortar in liquid nitrogen. A sharp blow with a steel pestle or hammer will fragment the material, which can then be ground to a fine powder in the frozen state and extracted.

From Blood Serum

Blood should be drawn by venipuncture or, in the case of small animals, by puncturing the heart or a suitable large blood vessel. The blood should be drawn into hypodermic syringes and immediately transferred to centrifuge tubes. Centrifugation (at 550 to 700 × g) to obtain the serum should be carried out in a refrigerated centrifuge at 4°C. The serum should be straw-colored and not show any signs of hemoly-

sis. Some investigators allow blood to clot before they draw off the serum, a process that may take several hours. This is not a good practice, since the lipids will oxidize.

Several procedures for the extraction of lipids from serum have been described in the literature, all of them similar. The one given here is a minor modification of the method used by Williams *et al.* (*2*).

The serum is extracted with chloroform-methanol using a ratio of serum:chloroform:methanol of 5:6:12 v/v/v. The appropriate volume of methanol is placed in a glass-stoppered Erlenmeyer flask. The serum is then added slowly, with mixing, to the methanol. The chloroform is added and the flask is placed in a water bath at 55°C for 30 minutes. The solution is filtered immediately through a Buchner funnel and the residue is washed with water:methanol:chloroform 5:6:12 v/v/v, using 1ml of wash solution per ml of original serum. The combined filtrates are allowed to separate into two phases in a separatory funnel at 4°C. The lower layer contains the lipid. The top aqueous layer should be free of lipid; this should be checked by concentrating the upper layer, running TLC plates (see Chapter 4), and looking for neutral lipids and phospholipids (see p. 45).

The solvent in the lower layer is then removed under vacuum. It is preferable to carry this out with a good vacuum pump rather than a water-type pump; this makes the application of heat unnecessary. If this is not possible, the heat should be kept to a minimum. The evaporating system (rotary type evaporator, flash evaporator, etc.) should be fitted so that nitrogen can be introduced into the system for controlling pressure and releasing the vacuum.

The lipid obtained should be redissolved in a minimum amount of chloroform-methanol and if necessary refiltered. Although the serum lipid is essentially subjected to a Folch-type washing procedure (*3*) in the above routine, some nonlipid material may still be carried through. Redissolving the sample in chloroform-methanol solution generally eliminates this.

The lipid sample is then placed in a dessicator over potassium hydroxide or some other dessicant and dried under vacuum. As noted earlier, the dessicator should be flushed out with nitrogen and evacuated a couple of times. When the weight of lipid is known, an antioxidant such as BHT can be added at the level of 0.1 percent of the weight of lipid. This weighing procedure is probably best when only very small amounts of lipids are being handled and when prevention of waste is imperative. There is the danger that the lipid may get exposed to air during weighing. It is essential to flush out containers with nitrogen and stopper them tightly during this time. When wastage is not an issue, the weighing procedure outlined by Rouser *et al.* (*4*) is recommended: the lipid is dissolved in a known volume of solvent and an aliquot (50 to 200 μl) is removed and weighed on a microbalance. This procedure is

not suitable if the lipid sample contains short-chain volatile fatty acids or their methyl esters.

Some authorities suggest adding the antioxidant to the extracting solvents so that the lipid is protected throughout the extraction procedure. This is a useful technique if one knows approximately the amount of lipid that will be extracted so that the appropriate amount of antioxidant can be added to the solvents. This procedure, however, necessitates either the determination of the amount of antioxidant in the sample or the accurate measurement of the solvents in order to obtain the weight of lipid.

As always with lipids, analyses should be completed as soon as possible. Lipids to which antioxidant has been added, however, can be stored in glass containers and under nitrogen at −20°C or below with little decomposition over fairly long periods.

From Erythrocytes

It is somewhat more difficult to extract the lipids from erythrocytes (or erythrocyte stroma preparations) than from serum. First, there is a tendency for the erythrocytes to form a rubbery mass that is difficult to break up, making consequent extraction inefficient. This can be prevented as follows. Place the appropriate amount of methanol in any conventional tissue homogenizer, add the cells, and homogenize. Then add the correct volume of chloroform and homogenize again. One extraction with this solvent mixture will not remove the lipids completely. The number of extractions and the variations in the solvent mixture will depend on the tissue involved. Rouser *et al.* (*4*) have proposed an exhaustive extraction sequence (the figures in parentheses refer to the milliliters of solvent per gram of fresh tissue):

1. Chloroform-methanol, 2:1 v/v, twice (20ml, then 10ml)
2. Chloroform-methanol, 1:2 v/v (10ml)
3. Chloroform-methanol, 7:1 saturated with 28 percent (by weight) aqueous ammonia[1] (10ml)
4. Chloroform-methanol-glacial acetic acid, 8:4:3 v/v/v (10ml)
5. Chloroform-methanol-concentrated hydrochloric acid, 200:100:1 v/v/v (10ml)

This complete extraction sequence is not required for efficient extraction from most tissues. In most cases the first three systems suffice, and frequently efficient extraction is obtained by extraction with chloroform:methanol 2:1 v/v only. In the case of erythrocytes, extraction with the first three solvents is recommended.

[1] Commercial preparations of 28% aqueous ammonia usually contain nonvolatile solids, largely silicates. To avoid this contamination, ammonia can be bubbled from a cylinder into ice-cold distilled water in a plastic container, in which the solution is then stored.

The combined solvent extracts will contain nonlipid material. This can be removed using the Folch washing procedure (*3*). In this method 0.2 volumes of water (or a salt solution) is added to the combined solvents in a separatory funnel and the nonlipid material passes into the aqueous phase. This procedure is widely used but is not entirely satisfactory since some loss of lipid occurs. As an alternative, most nonlipid material can be removed by one of the other methods described below.

The combined solvents freed of nonlipids and containing the erythrocyte lipids are removed under vacuum as previously described, and the lipid sample is dried and weighed.

When working with erythrocytes, fresh cells must be used, since in aging erythrocytes the lipids are rapidly oxidized by the catalytic effect of heme. This is also the reason for removing nonlipid material from the lipid sample and adding an antioxidant.

From Brain

Brain can be efficiently extracted with chloroform-methanol 2:1 v/v. Fresh or frozen brain should be placed in a suitable homogenizer or mortar and extracted three times with chloroform-methanol 2:1 v/v, using 20ml per gram of brain the first time and then 10ml per gram the remaining two times.

Brain contains many gangliosides that partition mainly into the top (aqueous) phase in the Folch washing procedure. Some gangliosides usually remain in the bottom phase, however, and any gangliosides entering the top phase may carry other lipid with them. The method is, therefore, not reproducible and if a total extract of brain lipids is required, aqueous washing should not be used.

Redissolving the lipid after removing the solvent is another way of removing nonlipid material. Provided that the solvent is removed at a low temperature and two phases exist during evaporation, any carried-over protein will be denatured. The solvents containing the extracted lipids plus nonlipid contaminants should be placed in a suitable flask for the evaporator system being used. If two phases are not present, add water until two phases are formed. Remove the solvents under vacuum, applying just enough heat via a water bath to prevent the formation of ice on the outside of the flask. The dried crude extract obtained can then be re-extracted three times with chloroform-methanol 2:1 v/v (1ml per 100mg of lipid) and the insoluble residue removed by filtration through a sintered glass filter (medium). Re-extraction and filtration can be carried out at room temperature.

Nonlipid material can also be removed by one of the column chromatographic procedures described later (pp. 27-29). When the final solvent is removed and the lipid dried, great caution must be used in subsequent handling. Brain lipid is rich in highly unsaturated (penta- and hexa-

enoic) fatty acids, which oxidize rapidly. An antioxidant should be used, and the lipid must be carefully protected from air.

From Other Sources

The extraction procedure employed for brain is suitable for extracting lipids from many soft tissues such as liver, spleen, kidney, adipose tissue, etc. With other tissue, however, individual problems are encountered and modifications must be made. Nerves, skin, heart, and muscle are not easily homogenized in the ground glass homogenizers of the Potter-Elvehjem type. Special handling such as grinding in a mortar or cutting up in a Waring Blendor may be necessary. The most useful technique with many tougher tissues is to grind the solidly frozen tissue to a powder as previously described (p. 22). Plant sources, particularly some leaves and seeds, frequently present similar problems and more severe grinding procedures must be employed.

If no established extraction procedure is available for a particular material, it should be subjected to the exhaustive extraction sequence previously described. Each solvent system should be evaporated off separately in order to determine how much lipid it extracted. In any event, solvent systems containing acid should be evaporated separately as the presence of acid may hydrolyse labile lipids. Finally, it may be necessary to subject the homogenized material to acid or alkaline hydrolysis and to check the hydrolysate for the presence of fatty acids. This will ascertain if any very tightly bound lipid remains unextracted. For example, the fatty acids in feces are present in the form of soaps; it is therefore necessary to treat feces with dilute (10 to 20 percent) hydrochloric acid before extracting. Problems of a similar nature are associated with some microbial sources in which fatty acids are found to be associated with glycoproteins. These sources of lipid constitute separate problems in themselves and, as special cases, are beyond the scope of this book.

REMOVAL OF NONLIPID CONTAMINANTS FROM EXTRACTS

Aqueous Washing

As noted earlier, hazards are associated with this procedure, and in certain cases, for instance when total brain lipid is required, its use is unwise. Although simpler, more reliable methods are now available, aqueous washing still enjoys wide acceptance, however, and objections to it are probably somewhat overstated. The method as described here applies to the combined 2:1 v/v chloroform-methanol extracts.

In this procedure the crude chloroform-methanol extract is washed with 0.2 its volume of water or a suitable salt solution. It is necessary to prepare solutions known as "pure solvents upper phase" and "pure solvents lower phase." These are prepared by mixing chloroform,

methanol, and water (8:4:3 v/v/v) in a separatory funnel. On standing, two phases separate. These are collected and stored in glass bottles. The upper phase consists of chloroform-methanol-water, approximately 30:48:47 v/v/v, and in the lower phase the proportions are 86:14:1 v/v/v. The phases may be prepared by using the above proportions if preferred. A "pure solvents upper phase" that contains 0.02 percent $CaCl_2$, 0.017 percent $MgCl_2$, 0.29 percent NaCl, or 0.37 percent KCl serves as the salt solution wash. The salt solutions may be prepared either by shaking the correct amount of salt with "pure solvents upper phase" or by replacing the water during the preparation of "pure solvents upper phase" with the following aqueous solutions: 0.04 percent $CaCl_2$, 0.034 percent $MgCl_2$, 0.58 percent NaCl, or 0.74 percent KCl. Using a salt solution rather than pure water reduces loss of lipids into the upper phase. Usually a crude lipid extract already contains salts, which helps conditions somewhat, but additional salts may improve the distribution required. Gangliosides, for example, found mainly in the upper phase in all systems, will be almost entirely eliminated from the lower phase by a $CaCl_2$ wash. This wash should not be used, however, if insoluble calcium salts are likely to be formed.

The washing procedure is the same for both the aqueous and the salt wash. Washing is carried out in a separatory funnel if the phases are to be allowed to separate by standing, or in a centrifuge tube if phase separation is to be achieved more rapidly by centrifugation (at approximately 600 × g).

The crude extract is mixed thoroughly with 0.2 its volume of water or one of the salt solutions. When the biphasic system is obtained, as much of the upper phase as possible is removed by siphoning. The interface is then rinsed three times with small amounts of "pure solvents upper phase." The rinsing should be done carefully so as to avoid disturbing the lower phase. After the last rinse, the lower phase and any unremoved rinse solution are combined into one phase by adding methanol. The solution can then be diluted with methanol to any desired volume, or the solvent can be removed under vacuum and the lipid dried.

Treatment on Cellulose and Sephadex Columns

Rouser *et al.* (*5*) describe a cellulose column chromatographic procedure which removes water-soluble nonlipid contaminants from crude lipid extracts. Gangliosides are eluted with the nonlipid fraction.

The column size chosen will depend on sample size. The procedure described below is suitable for a 100 to 500mg sample.

A glass column 2.5cm internal diameter (i.d.) × 20 to 30cm long is packed with 20g of Whatman standard grade ashless cellulose powder suspended in methanol-water 1:1 v/v (see p. 31 for hints on packing glass columns). The packed bed is washed at a flow rate of 3 ml/min with the following set of solvents: methanol-water 1:1 v/v (7 column

volumes[2]), chloroform-methanol 1:1 v/v (3 column volumes), and chloroform-methanol 9:1 v/v saturated with water (4 column volumes).

The sample, 100 to 500mg, is added to the column in 5 to 10ml of the last washing solution. Two fractions are then eluted: the first, with chloroform-methanol 9:1 v/v saturated with water (20 column volumes), contains lipids minus the gangliosides, if present; the water-soluble nonlipid contaminants and the gangliosides are then eluted with 12 column volumes of methanol-water 9:1 v/v.

Sephadex, a cross-linked dextran gel, can also be used to separate lipids and water-soluble nonlipids. Procedures have been described by Wells and Dittmer (*6*) and by Siakotos and Rouser (*7*). The latter method is described below.

Four solvent systems are required (all proportions are by volume):

1. Chloroform-methanol 19:1, saturated with water
2. 850ml chloroform-methanol 19:1 plus 170ml glacial acetic acid plus about 25ml water (to saturate)
3. 850ml chloroform-methanol 9:1 plus 170ml glacial acetic acid plus about 42ml water (to saturate)
4. Methanol-water 1:1

When preparing solvent mixtures 1, 2, and 3, shake them vigorously and allow them to separate in a separatory funnel. The lower phase of each is used for chromatography. When adding the water to mixtures 1, 2, and 3, do so slowly so that the mixtures are *slightly* undersaturated.

Sephadex (G-25, coarse, beaded, Pharmacia Fine Chemicals, New Jersey) is placed in methanol-water 1:1 v/v. The "fines" in the material are removed by decantation and dissolved gases are removed by gentle suction from a vacuum pump. This takes about 1 minute. After equilibrating at room temperature for several hours, the gel is again degassed and poured into a suitably sized column. For samples of 50 to 250mg, a column 1cm i.d. × 10 to 30cm is appropriate. A packed 2.5cm i.d. × 30cm column will accommodate several grams of sample. To prevent the gel from floating in the solvents add a 2.5 to 5cm layer of clean sand above it.

Transfer the sample onto the column in solvent mixture 1 and cover the top of the sand with a plug of washed glass wool. For the smaller samples on the 1cm i.d. × 10cm columns elute with the following volumes of the solvents: 1, 25ml; 2, 50ml; 3, 25ml; and 4, 50ml. The flow rate should be 3ml per minute. Larger samples up to 5g on the 2.5cm i.d. × 30cm columns should be eluted with 20 times the above volumes.

[2] A column volume (sometimes called the bed volume) is the volume of the column occupied by the adsorbent.

Depending on the complexity of the lipid-nonlipid mixture, the four fractions may contain a variety of substances. The following is a comprehensive list of the substances found after chromatography of samples from various sources (*5*).

Fraction 1: Hydrocarbons; mono-, di-, and triglycerides; sterols; sterol esters; waxes; all phosphoglycerides (including lyso compounds); sulfatides; cerebrosides; sulfolipids; free fatty acids; glycosyldiglycerides; unconjugated bile acids; conjugated bile acids (glycine, taurine); and uncharacterized nonlipid compounds. The unconjugated bile acids are eluted whether they are applied as salts or free acids and the conjugated when applied in the free acid form (*4*). The bile acids and related steroids are not covered in this text; any major biochemical text (such as *8*) gives accounts of the steroids.

Fraction 2: Gangliosides; glycine conjugated bile acids (applied as salts); some acidic phosphatides (in "altered" form); urea; and other materials soluble in organic solvents.

Fraction 3: Traces of gangliosides; taurine-conjugated dihydroxycholanic (deoxy and chenodeoxy) acids (when applied as salts); some amino acids; and other uncharacterized organic substances.

Fraction 4: Taurocholate and most water soluble nonlipids such as salts, amino acids, sugars, etc.

Note that, for the purposes of the types of analyses covered in this text, Fraction 1 contains the bulk of the lipids, while Fraction 2 is rich in gangliosides.

This procedure is the most efficient and reliable method now available for the removal of water-soluble nonlipids from crude lipid extracts. It is also relatively simple to set up and use. Columns can be reused after allowing them to stand in methanol-water 1:1 (solvent mixture 4) for about 48 hours, then washing with 100 to 500ml of solvent mixture 1 immediately before use.

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III. Column Chromatography

THE CHROMATOGRAPHIC TECHNIQUE has been used by organic chemists as a separation method for many years. Early developments were confined to column and paper chromatography and the types of separations achieved were limited. However, in 1952 the first gas-liquid chromatograph (*1*) was introduced, and in 1958 Stahl (*2*) described his development of thin-layer chromatography. Rapid development of these two techniques, together with improved applications of column chromatography, has revolutionized the field of lipid analysis. New and improved methods are still being reported in some abundance, from which we have selected a few well-tested procedures in each area of chromatography. These can be applied with good reproducibility to the analysis of mixtures of neutral, phospho-, and glycolipids. Separations of the rarer lipids and of complex lipids such as gangliosides still present some difficulties, as does the separation of individual molecular species of lipid classes. The major lipids of blood serum, say, can be analyzed, including their fatty acid and fatty alcohol patterns, provided that the indicated procedures are followed strictly and the lipids are carefully protected from oxidation and contamination. This introduction to chromatographic techniques is geared to that level of achievement. It should, however, be stressed that in many respects chromatography is very much an art, requiring practice before effective separations can be obtained.

Chromatography includes any technique in which compounds are physically separated by differential distribution between two phases, one of the phases being stationary and the other moving. Stationary phases are either solid or liquid and moving phases either liquid or gaseous.

In column chromatography the stationary phase is packed in a glass column onto which the sample is introduced; the moving phase is liquid. Separation is achieved by percolating suitably constituted solvents through the bed of stationary phase. Column chromatography can be subdivided into two main types according to the nature of the stationary phase: solid-liquid adsorption chromatography and liquid-liquid partition chromatography.

SOLID-LIQUID ADSORPTION CHROMATOGRAPHY

This type of chromatography is based on the differences in affinity of compounds for the solid adsorbent that serves as the stationary phase. The relative affinity of compounds for the phase depends upon the nature of their polar groups and to some extent upon the van der Waals forces exerted by their nonpolar groups. The greatest forces involved

in the affinity for the adsorbent are those due to polar and ionic groups. This means that this type of chromatography is useful for separating lipid mixtures which differ in the number and type of their polar groups. Thus, broad separations of the neutral lipids from the main types of polar lipids can readily be carried out. Separations within lipid classes can also be achieved. Many adsorbents have been employed, among them alumina, magnesium oxide, urea, Florisil (a magnesia silica gel), silicic acid, diethylaminoethyl cellulose (DEAE), and others. The most efficient and versatile separations to date have been obtained using DEAE. Silicic acid is, however, very useful for separation of lipid classes, and Florisil is especially useful for the separation of cerebrosides. The procedures given utilize these three main adsorbents.

A typical adsorption chromatography set-up is shown in Figure 1. This is the simplest form of apparatus which should be employed. Note the Teflon stopcocks (to avoid use of grease). Also note the nitrogen line so that a slight positive pressure of nitrogen can be maintained over the system.

LIQUID-LIQUID PARTITION CHROMATOGRAPHY

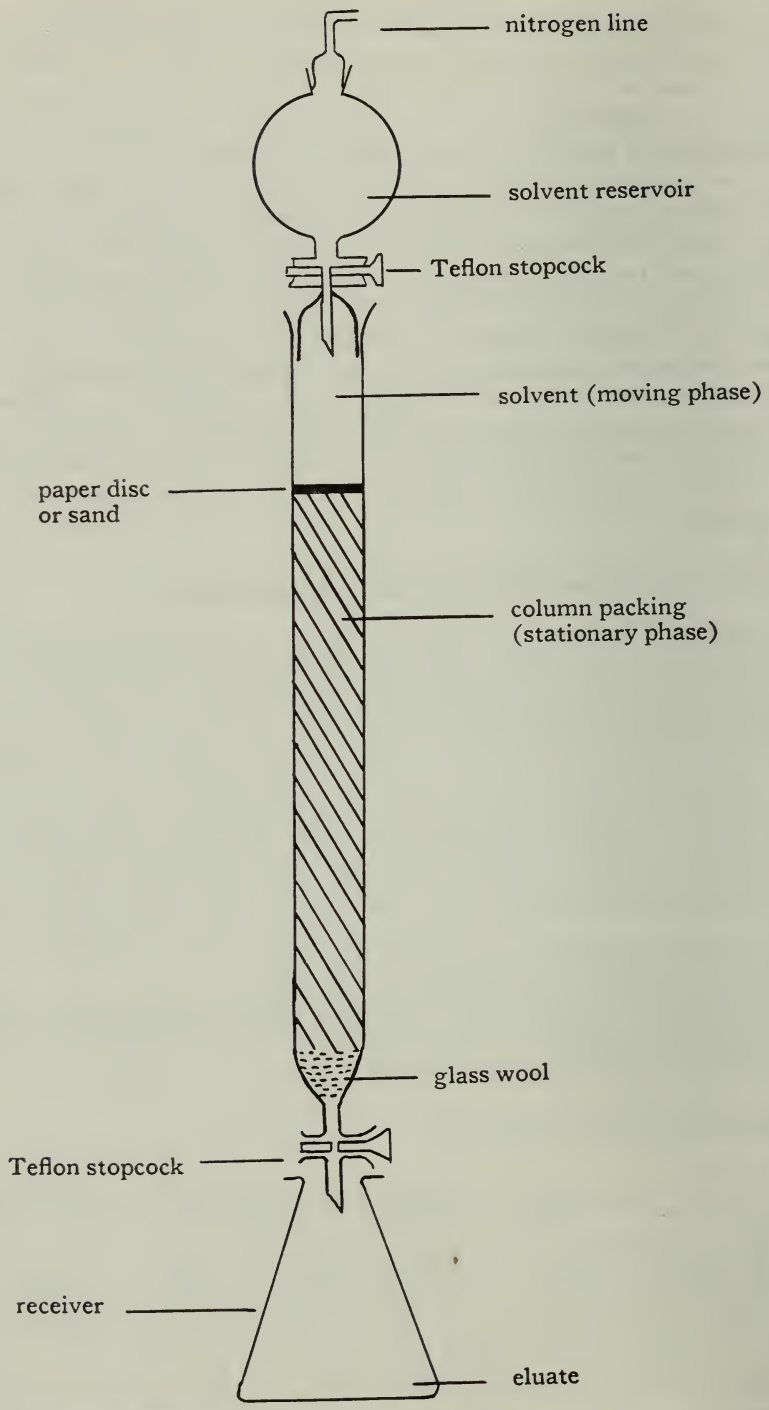
Liquid-liquid partition chromatography is essentially a countercurrent distribution process. In this method, separation depends on the relative solubility characteristics of the compounds with respect to the stationary and moving phases of the column. Two types of liquid-liquid columns are in general use, one in which the more polar liquid is stationary (partition type) and the other in which the less polar liquid is stationary (reversed-phase partition type). Liquid-liquid partition chromatography has been used largely for the separation of fatty acids. Separations have been obtained according to unsaturation, chain length, and geometrical isomerism. One of the major uses of this method (especially when preparative gas chromatography is not available) is the large-scale preparative separation of fatty acids.

We shall be concerned only with adsorption chromatography. Before describing some separations it is essential that we consider the basic principles of column packing and preparation.

PREPARATION OF COLUMNS

Separation and reproducibility depend on a number of factors, the most important of which are packing of the column, pretreatment of the adsorbent, shape and dimensions of the column, rate of elution, quality of the eluting solvents, and load of sample in relation to weight of adsorbent. Temperature and humidity also influence reproducibility.

It is usually best to introduce the adsorbent into the column in the form of a thin slurry or suspension. The slurry is prepared by adding the desired weight of packing material to a suitable volume of solvent. The solvent used is generally the one with which the adsorbent is to be



Apparatus for adsorption column chromatography. (Fig. 1

prewashed or pretreated; in some instances, the first eluting solvent may be used. A plug of glass wool should first be placed in the shoulder of the column (Fig. 1). As the adsorbent is poured in and allowed to settle, the flow of solvent through the column is kept constant. This procedure prevents the formation of "channels" in the column, a common hazard when columns are packed dry. Channel formation is quite disastrous in column chromatography, since it leads to very inefficient separation and overlapping of fractions. Applying pressure to pack columns is a dangerous practice and generally does not prevent channeling. If pressure is applied to a glass column, which is hardly designed as a pressure vessel, the glass may break and possibly cause injuries.

Pretreatment of the adsorbent varies according to the adsorbent being used. In the examples which follow, therefore, pretreatment is, dealt with individually. It is essential that a recommended pretreatment is carried out, as frequently this step endows the adsorbent with its particular characteristics.

The shape and length of columns affect separation; column dimensions, therefore, should always be stated. It has been demonstrated that longer, narrower columns usually give better separations than shorter, wider ones (*3*). If a column is tried and it is found that some fractions overlap, increasing the column length often solves the problem. Extremely long columns are, of course, inconvenient; increasing the length beyond 40cm is not recommended.

Once elution is started, a flow of nitrogen through the column (preferably via a proper flow control) can be maintained as an antioxidation precaution.

While investigators invariably recommend a particular sample load for best resolution, the same load may not be optimal in all laboratories. Slight differences in the adsorbent as well as in environmental factors such as laboratory temperature and humidity may affect resolution. Therefore, even though a method may be well established, it may be necessary to change some dimensions of the procedure if resolution is not satisfactory. This is done by applying different loads to a particular column until the best resolution is obtained. Ideally, and in the interests of saving time, one should strive to ascertain the maximum load that can be applied to the smallest column (that is, the column with the smallest elution volume) to give satisfactory resolution of fractions. The composition of fractions and the degree of resolution obtained are very readily checked by thin-layer chromatography. This technique and its use as an adjunct to column chromatography are discussed in the next chapter.

The quantitative analysis of samples by column chromatography can be accomplished in a number of ways, depending upon the composition of the sample and the needs of the investigator. It is usual to analyze each tube of eluate for a suitable element or functional group and to

plot its concentration versus tube number. Each tube should contain a constant volume of eluate (usually 5 or 10ml), which may be collected manually or by one of the many available automatic fraction collectors. In making a preliminary separation of, say, cholesterol and glycolipids from phospholipids, each tube would be analyzed for phosphorus.

Once the elution pattern has been established, an appropriate large volume of eluate may be collected, concentrated to a known volume, and checked by thin-layer chromatography for composition. A known aliquot can then be analyzed for the appropriate element or compound and the total amount in the fraction calculated. Contents of each lipid can be reported in a variety of ways, such as mg lipid phosphorus per g of total lipid, or per g of wet — or dry — tissue. Examples of the calculation of such expressions are given in the final chapter. Sometimes merely a weight of a fraction may be required; this may be done directly by evaporating the solvents to a known volume, taking an aliquot, removing the solvent, and weighing on a microbalance. Thin-layer chromatographic checks of fraction content should still be made for every run.

Silicic Acid Columns

For some time silicic acid and alumina were the only adsorbents used for the chromatography of lipids. Both adsorbents have to some extent been supplanted by others, but silicic acid is still used, especially for initial broad separations. Fine-degree resolution requires rechromatography on other adsorbents.

Early separations on silicic acid were done using fine mesh preparations. As a consequence, flow rates were very slow and the procedures time-consuming. Reproducibility of results on silicic acid columns is not easily achieved since different batches of the adsorbent may vary considerably in their properties. To some extent, these disadvantages can be overcome by using one of the commercially available silicic acids that have been manufactured with a view to maintaining a uniform standard grade (for example, Adsorbosil, Applied Science Laboratories, and Unisil, Clarkson Chemical Company). Using these preparations in coarser mesh sizes overcomes the problem of slow flow rates. Using standardized silicic acid preparations also gives better reproducibility, although reproducibility between one laboratory and another remains difficult. The procedures that follow are, therefore, generalized. Each investigator must standardize procedures according to his individual needs, and each lot of adsorbent must be separately assessed.

Usually the better grades of silicic acid do not require pretreatment, but they should be activated at 120°C if they have been in a moist atmosphere. Activation is necessary to remove some of the absorbed moisture, which could affect the adsorption properties of the silicic acids.

The column in general use is 2.5cm i.d. and 10 to 20cm long; the sample loading for separations of neutral from phospholipid classes is about 10 to 30mg per g adsorbent.

The mechanisms involved in silicic acid chromatography and the elution patterns of lipids on this adsorbent have been discussed in great detail by Wren (*4*). Wren gives an expanded version of Trappe's "eluotropic series" (solvents listed in order of their eluting power). The solvents as listed by Wren are: methanol; ethanol; 1-propanol; acetone; methyl acetate; ethyl acetate; ether; dichloromethane; benzene; toluene; 1,1-dichloroethane; 1,1,2,2-tetrachloroethane; chloroform; trichloroethylene; carbon tetrachloride; cyclohexane; and petroleum ether of various boiling ranges. Of these solvents those most frequently used are methanol, ether, chloroform, and petroleum ether. Wren (*4*) also listed the approximate order in which one may expect lipids to be eluted from a silicic acid column: hydrocarbons, esters (other than sterol esters and glycerides), sterol esters, fatty aldehydes, triglycerides, long-chain alcohols, fatty acids, quinones, sterols, diglycerides, monoglycerides, glycolipids, lipamino acids, bile acids, glycerophosphatidic acids, inositol lipids, phosphatidyl ethanolamines, lysophosphatidyl ethanolamines, phosphatidyl cholines, sphingomyelins, lysolecithins.

Initial separation: neutral from phospholipids and glycolipids. When attempting to separate naturally-occurring lipids, it is best to start by carrying out a crude separation into neutral lipids, phospholipids, and glycolipids. Like all the column chromatographic procedures described here, this is done using a stepwise elution.[1] The sample (100 to 150mg per g of adsorbent) is added to a column of coarse mesh silicic acid. Elution is carried out first with chloroform (10 column volumes for a 2.5cm i.d. × 5cm column). This elutes neutral lipid. Ten column volumes of chloroform-methanol 1:1 v/v or of methanol alone will elute the phospholipids. If the lipid sample contains a lot of glycolipids, an intermediate elution with acetone (30 to 50 column volumes) will elute these as a separate fraction before the phospholipids. Cardiolipin will be largely eluted in an acetone fraction along with the glycolipids. The three-step elution is very useful for the preliminary separation of nervous tissue lipids, in which the first fraction will consist almost entirely of cholesterol.

Separation of neutral lipids. Numerous solvent systems have been used for the separation of neutral lipids on silicic acid. The choice of procedure will depend on the composition of the fraction and the

[1] Better resolutions can often be obtained by using gradient elution, which frequently overcomes tailing of one lipid fraction into another; however, stepwise elution is simpler and needs no special equipment. Automatic equipment for the mixing of solvent gradients can be obtained commercially. Some references to gradient elution procedures occur in the text.

relative proportions of the constituents. A procedure to suit most needs should be found among the following:

Hydrocarbons (saturated) can be eluted first with light petroleum ether, hexane, or Skelly B. Unsaturated hydrocarbons such as squalene will usually be the tail of this fraction.

Esters of fatty acids, cholesterol esters, and wax esters are eluted together with 1 percent diethyl ether in petroleum ether (*5*). Esters containing hydroxylated fatty acids require a more polar solvent for their elution, a fact that can be used to separate nonhydroxylated and hydroxylated fatty acids and their esters. For example, pentane with 3 percent diethyl ether elutes nonhydroxylated methyl esters, and pentane with 20 percent diethyl ether elutes the hydroxylated form (*6*). Free acids can be separated into nonhydroxylated and hydroxylated groups by eluting first with benzene, then with benzene-diethyl ether 9:1 v/v (*7*).

Fatty acid esters which differ in degree of unsaturation can be separated on silicic acid by various procedures. Goldfine and Bloch (*8*) and Erwin and Bloch (*9*) achieved separation of fatty acids according to degree of saturation by treating the methyl esters with mercuric acetate and chromatographing the mercuric adducts on silicic acid. The saturated esters were eluted with pentane-ether 95:5 v/v, the monoenoic esters with ethanol-ether 50:50, the dienoic with ethanol-acetic acid 99:2, the trienoic with ethanol-acetic acid 99:1, and the tetraenoic and polyenoic esters with ethanol-acetic acid 95:5.

Silicic acid impregnated with silver nitrate has been used by DeVries (*10*) to separate saturated and unsaturated fatty acid methyl esters and their geometric isomers. DeVries (*11*) also employed this absorbent to separate triglycerides according to their degree of unsaturation. See also pages 51-52.

Triglycerides separate from wax and sterol esters on silicic acid by eluting with 3 to 5 percent ether in petroleum ether. Free fatty acids and fatty alcohols are eluted after triglycerides with a slightly more polar solvent, but tailing into the triglyceride fraction is usual. Diglycerides elute with 20 to 60 percent ether in petroleum ether, and monoglycerides elute with ether or chloroform (*5*). Monoglyceryl ethers accompany the monoglyceride fraction.

A hazard is encountered when chromatographing monoglycerides on silicic acid: both the 1- and 2-isomers isomerize. Diglycerides do not isomerize (*5, 13*). Isomerization of monoglycerides is prevented by using silicic acid impregnated with 10 percent w/w boric acid (*12*). On this packing, the 1- and 2-monoglycerides can be efficiently separated.

Separation of polar lipids. Polar lipids cannot be completely separated on silicic acid, but some separations are possible by eluting with increasing amounts of methanol in chloroform. Much better reso-

lutions, however, can be obtained using diethylaminoethyl cellulose (DEAE), described below.

Florisil Columns

Florisil (Floridin Co., Pennsylvania Glass Sand Corp., 2 Gateway Center, Pittsburgh, Pennsylvania) is a synthetic magnesium silicate. It has a number of uses in lipid separation, the chief one being the separation of cerebrosides, sulfatides, and ceramides. Florisil has the disadvantage, however, that its chromatographic properties are changed when traces of water are present. This problem may be overcome by drying the adsorbent and by using very dry solvents. Solvents may be dried by adding 5 percent 2,2-dimethoxypropane to them as described below. A useful procedure for the preparation of ceramide, cerebrosides, and sulfatides, described by Rouser *et al.* (*14*), is outlined here.

One pound of Florisil is washed on a sintered-glass filter (medium porosity) with 8 bed-volumes of water. It is then activated at 100°C for 6 hours and cooled without exposure to air. The appropriate amount of Florisil is quickly weighed and 1 percent (by weight) water is added. Let the mixture stand for an hour or more in a closed container so that the water and adsorbent will equilibrate. Mix the equilibrated adsorbent with chloroform containing 5 percent (by volume) 2,2-dimethoxypropane. This slurry is then poured into a chromatographic tube.

A 10.2cm i.d. column is used for the separation of ceramides, cerebrosides, and sulfatides from about 3g of brain lipid. The lipid sample is placed on the column in chloroform containing 5 percent 2,2-dimethoxypropane. The eluting solvents, which must all also contain 5 percent 2,2-dimethoxypropane, are:

1. Chloroform, 10 column volumes
2. Chloroform-methanol 19:1 v/v, 10 column volumes
3. Chloroform-methanol 70:30 v/v, 20 column volumes

The first fraction will consist of cholesterol and any less polar lipids. The second will be ceramides, and the third, cerebrosides and sulfatides. The flow rate of solvents should be about 50ml per minute. If the phosphatides are to be collected, they can be eluted last with 20 column volumes of chloroform-methanol 2:1 v/v, saturated with water. The ceramides can be purified by preparative thin-layer chromatography (see next chapter), and the cerebrosides and sulfatides can be separated on a DEAE column.

Diethylaminoethyl (DEAE) and Triethylaminoethyl (TEAE) Cellulose Columns

DEAE cellulose gives the most satisfactory column chromatographic separations of complex lipid mixtures. Separation is achieved either

by ion exchange or by differences in polarity of nonionic groups. The elution characteristics of DEAE are most easily understood if lipids are considered as being in three groups — nonionic, nonacidic ionic, and acidic. Nonionic lipids are eluted according to relative polarity. The least polar lipids such as the sterols, sterol esters, glycerides, and hydrocarbons are eluted with chloroform. Three to 5 percent methanol in chloroform elutes cerebrosides and glycosyl diglycerides. All of the nonionic and ionic nonacidic lipids are eluted with increasing amounts of methanol in chloroform. The acidic lipids, however, are not eluted with chloroform-methanol or methanol unless an acid, base, or salt is added to the solvent.

The development of DEAE cellulose column chromatography is due largely to Rouser and his co-workers, who have compiled an extensive review and detailed description of all aspects of this method (*14*). Here we shall deal with preparation and pretreatment of columns and typical elution sequences for the more common lipids. For variations and separations of more complex mixtures, the Rouser article should be consulted.

The DEAE cellulose usually recommended is Selectacel DEAE cellulose regular grade (Brown Co., Berlin, New Hampshire). This is a coarse grade, which is usually best for lipid separations. The DEAE must be washed before use, as it contains impurities. Washing can be done on a sintered-glass or Buchner funnel, using about 3 bed volumes of acid or base. The DEAE is washed first with 1N hydrocloric acid, rinsed with water to a neutral pH, then washed with 0.1N aqueous potassium hydroxide followed by a water rinse to neutral pH again. After washing, the DEAE is converted to the acetate form by washing it with 3 bed volumes of glacial acetic acid. The adsorbent is air-dried, then dried to constant weight in a vacuum dessicator over potassium hydroxide. Thorough drying is important. The dry DEAE is left overnight in glacial acetic acid and finally is added to the column as a slurry in glacial acetic acid. The excess acid is blown through the column with nitrogen, and the adsorbent is patted down gently with a glass rod. A 30cm column, 1.0 to 4.5cm i.d., is a useful size. The packing should be about 20cm high. After packing, the bed of adsorbent is washed with 3 bed volumes of methanol, 3 bed volumes of chloroform-methanol 1:1, and 3 to 5 bed volumes of chloroform. The packing should not be allowed to run dry at any time. The packed, washed column should then be tested. The recommended testing procedure is as follows:

To a column (2.5cm i.d. × 20cm) add a solution of 10 to 30mg of cholesterol in 5 to 10ml of chloroform. Collect 10ml volume fractions using chloroform as the eluting solvent (flow rate about 3ml/min.) and test each fraction for cholesterol. The best test is to add 5 drops of acetic anhydride and 1 drop of concentrated sulfuric acid to 1ml of fraction. A green-blue color indicates cholesterol. The column is judged satisfactory if cholesterol first appears in tube 7 to 9. If satisfactory,

the remainder of the cholesterol can be eluted with chloroform and the sample applied.

There are many possible elution schemes depending on the separations desired. Many elution sequences have been described by Rouser and co-workers (*14, 15*). Two of more general use are described below.

Separation of lipid samples into acidic and nonacidic fractions (*14*). Using a 6cm long and 4.5cm i.d. column of DEAE prepared in chloroform-methanol 2:1 v/v, apply the sample in the same solvent, then elute with the following sequence at a flow rate of 10ml/min.:

1. Chloroform-methanol 2:1 v/v (4 column volumes)
2. Methanol (10 column volumes)
3. Chloroform-methanol 4:1 v/v made 0.01 to 0.05M with respect to ammonium acetate, to which is added 20ml of fresh 28 percent w/w aqueous ammonia[2] per liter (10 column volumes)
4. Methanol (10 column volumes)

The first two fractions will contain all nonacidic lipids plus salts. The third fraction will contain acidic lipids plus salts, and the fourth will contain salts and possibly traces of lipids. The nonacidic lipids include all the neutral lipids and nonionic glycolipids (like the cerebrosides and phospholipids) that lack a negative charge. Acidic lipids include all those having only negatively charged groups, such as fatty acids and cerebroside sulfates, and those lipids that have at least one more negative group than positive groups, such as phosphatidyl serine.

A general elution scheme (*14*). This procedure utilizes a 20cm high, 2 to 5cm i.d. column of DEAE prepared in a slurry with choloroform. The sample is applied in chloroform, and the following solvents are used at a flow rate of 3ml/min.

1. Chloroform (10 column volumes)
2. Chloroform-methanol 9:1 v/v (9 column volumes)
3. Chloroform-methanol 7:3 v/v (9 column volumes)
4. Chloroform-methanol 1:1 v/v (9 column volumes)
5. Methanol (10 column volumes)
6. Chloroform-glacial acetic acid 3:1 v/v (10 column volumes)
7. Glacial acetic acid (10 column volumes)
8. Methanol (4 column volumes)
9. Chloroform-methanol-ammonium salt (as described in step 3 of the preceding procedure) (10 column volumes)
10. Methanol (10 column volumes)

Composition of the resulting fractions can be expected to be:

1. Neutral lipids
2. Cerebrosides, lysophosphatidyl choline, phosphatidyl choline, sphingomyelin, and mono- and diglycosyl diglycerides

[2] See footnote, p. 24.

3. Ceramide, aminoethylphosphonate, ceramide dihexosides and polyhexasides, and phosphatidyl ethanolamine
4. Ceramide polyhexosides, lysophosphatidyl ethanolamine, and uncharacterized oxidation products
5. Oxidation products and salts
6. Free fatty acids, glycine conjugated bile acids, and unconjugated bile acids
7. Phosphatidyl serine
8. Lipid-free fraction
9. Cardiolipid, phosphatidic acid, phosphatidyl glycerol, phosphatidyl inositol, sulfolipid, sulfatides, and salts
10. Salts and traces of lipid

Of course, not all the lipids listed above are likely to be present in one sample, and in most cases fractions will contain only one or two major components. Thus, a sample of serum lipid would yield the following fractions:

1. Neutral lipids
2. Lysophosphatidyl choline, phosphatidyl choline, and sphingomyelin
3. Phosphatidyl ethanolamine
4. Phosphatidyl serine
5. Phosphatidyl inositol

The first fraction could readily be separated on a silicic acid column into glycerides, sterol esters, and free fatty acid fractions. Separation of the second fraction would be best achieved by chromatography on a silicic acid-silicate column. The latter column is prepared by treating silicic acid with aqueous ammonia. Silicic acid (100 to 200 mesh) is placed on a coarse sintered glass filter and washed first with 3 bed volumes of 6N hydrochloric acid, then with 5 bed volumes of water. The adsorbent is then heated at 120°C for 6 hours and cooled without exposure to air. Using a 2.5cm i.d. column, a slurry of 25g of the silicic acid is prepared in 40 to 100ml chloroform-methanol 1:1 v/v and 4ml of 28 percent aqueous ammonia. The chromatography tube is filled to a height of 10cm. Before the sample is added, the column is washed with 4 column volumes of chloroform. The fraction containing phosphatidyl choline, lysophosphatidyl choline, and sphingomyelin (50 to 75mg) is added to the column in about 5ml of chloroform.

The following elution sequence is then applied:

1. Chloroform-methanol 4:1 plus 1.0 percent (by volume) water (8 column volumes)
2. Chloroform-methanol 4:1 plus 1.5 percent (by volume) water (11 column volumes)
3. Methanol plus 2 percent (by volume) water (5 column volumes)

Fraction 1 will contain the phosphatidyl choline, fraction 2 the sphingomyelin, and fraction 3 the lysolecithin.

Even the most complex mixtures of lipids can be separated by chromatography on DEAE when followed by suitable rechromatography of mixed fractions on silicic acid, silicic acid-silicate, Florisil, DEAE, or TEAE columns. Rouser *et al.* (*14, 15*) should be consulted for elution sequences suited to separation of complex mixtures.

The triethylaminoethyl (TEAE) cellulose differs from DEAE in having a greater capacity for lipids that have carboxyl groups as their only ionic group. Thus, it is an ideal choice for the separation of mixtures containing large proportions of fatty acids, gangliosides, and bile acids. TEAE is usually used in the hydroxyl form, and elution sequences similar to those used with DEAE are generally employed (*14*).

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IV. Thin-Layer and Paper Chromatography

THIN-LAYER CHROMATOGRAPHY

Although the basic principle of thin-layer chromatography (TLC) was first described many years ago, it did not become a popular technique until Stahl (*1*) reported his standardization of procedures.

In TLC the adsorbent is spread in a thin, even layer on a glass plate, and the components of an applied sample are separated by allowing a suitably composed solvent mixture to move through the adsorbent. The technique is simple and, in many cases, more rapid than others. The speed with which many separations can be achieved make TLC a most useful adjunct to column chromatography. It is relatively simple to apply a concentrated aliquot of solvent from a column chromatographic fraction to a TLC plate, develop the plate, and by suitable detecting agents determine the composition of the fraction. TLC is also useful in many instances as a purification procedure. Moreover, TLC enjoys wide popularity as an analytical tool in its own right; frequently the analysis of very small samples, which would be extremely tedious or impossible by other procedures, can be readily achieved by TLC. The technique is not without hazards, however, and they will be noted as each stage of TLC procedure is discussed.

Preparation of Thin-Layer Chromatographic Plates

Two sizes of glass plates are in general use: 4cm × 20cm and 20cm × 20cm. While plates can be obtained commercially as matched sets, it is usually more economical to have plates cut from plate glass and smooth their edges. Plates should then be marked, on the basis of evenness of thickness, as matched sets (5 large plates per set is useful for most spreading devices).

The plates should be thoroughly cleaned. Just prior to use they should be cleaned with a suitable solvent, such as chloroform or benzene, to remove any traces of grease that would interfere with the spreading of the adsorbent.

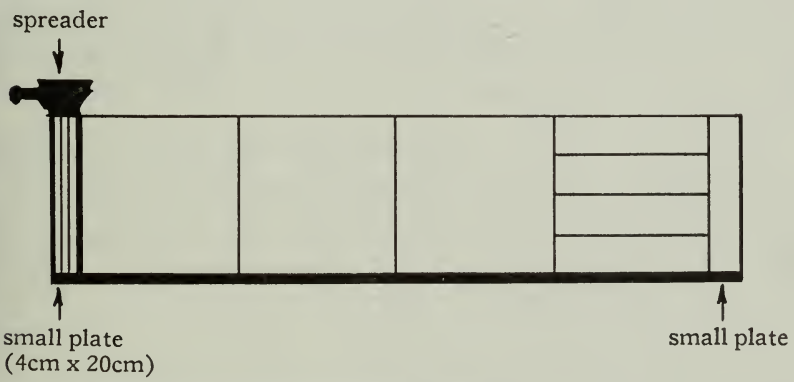
Several types of adsorbent are available. Some satisfactory ones for universal use are the Merck Silica Gel G (with and without calcium sulfate as binder), Merck Silica Gel H, and Supelcosil 12 B (Supelco, Inc.). Rouser and co-workers (*2*) recommend the use of Silica Gel G plain (no binder) plus 10 percent by weight magnesium silicate (Allegheny Industrial Chem. Co., P.O. Box 786, Butler, New Jersey) for chromatography of polar lipids.

Sometimes it may be preferable to wash adsorbents before use since impurities in them may interfere with subsequent analyses. A bulk

washing procedure for Silica Gel H, as described by Parker and Peterson (*3*), is recommended. The Silica Gel H (125g) is weighed and placed in a Buchner funnel on 2 pieces of Whatman No. 2 filter paper. Over this is poured 1 liter of a mixture of formic acid (98 to 100 percent), chloroform, and methanol 1:2:1 v/v/v. This is sucked through by water pump vacuum, and the bed of Silica Gel H is then washed with 500ml of deionized water. The adsorbent is then spread out on an enamel or heavy aluminum foil tray and dried in an oven at 110 to 120°C for 48 hours. The lumps should be broken up after 24 hours. When preparing a slurry from washed adsorbent, mix thoroughly in a Waring Blendor.

The one disadvantage of washing procedures of this type is that some characteristics of the gel change, and frequently it is difficult to prepare smooth, even plates. Usually it is more convenient to wash the adsorbent when it has already been plated. This is done by allowing a solvent system to develop beyond the planned solvent front. Chloroform-methanol-water, 65:25:4 v/v/v is a suitable solvent in most cases.

While TLC plates can be prepared by dipping and spraying, by far the most successful and reproducible method is spreading, using a commercial spreader and tray such as the Desaga (Brinkmann Instruments, Inc., 115 Cutter Mill Road, Great Neck, Long Island, N.Y.). The plates are aligned on the tray and a slurry of the adsorbent is placed in the spreader at one end of the board (Fig. 2). The slurry will vary in consistency according to the thickness of the layer desired. A suitable thickness for general use is 0.25mm. A fixed thickness 0.25mm spreader is available; otherwise, the variable thickness spreader is set accordingly. A slurry is prepared by mixing 20g of adsorbent thoroughly in about 65ml water. Note (Fig. 2) that a small plate is placed at each end of the board to allow for run-out from the spreader



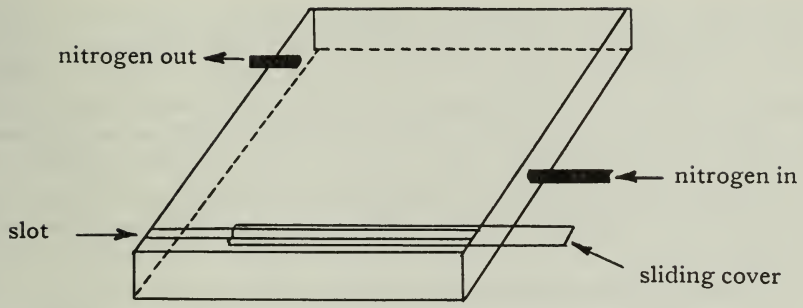
A thin-layer chromatography tray and spreader. **(Fig. 2)**

when it is opened and closed. These end plates are unevenly spread and therefore are not used. The lever on the spreader is turned through 90° and the spreader is moved steadily across the plates to the end, where the lever is again turned through 90° to close. The speed with which the spreader moves across the plates affects the thickness of the layers, and some practice is required before plates of uniform thickness are obtained. After the slurry has been applied, the spreader is removed and washed. The plates are then vibrated slightly by bouncing the tray up and down several times. This helps to even out the layers. The plates are air dried and then dried at 120°C for 30 minutes to an hour in a clean atmosphere free of vapors. It is best to prepare and use TLC plates under standardized conditions; otherwise, separations will not be reproducible from one day to the next. If the atmosphere is very humid, it may be necessary to treat plates in a humidifying chamber to ensure a more constant water content.

Before applying samples to plates, the edges should be made uniform by stripping the first 4 or 5mm of adsorbent in a straight line. If plates are to be developed in only one dimension, it is wise to divide the plate into lanes by scoring through the adsorbent. This prevents lateral spread of components towards each other.

Samples may be applied either as spots or streaks. They are usually applied with Hamilton Syringes of 10 to 50μl capacity (Hamilton Co., P.O. Box 307, Whittier, Calif.). It is usual to apply samples as a short streak (1cm) made by overlapping several small spots or as a single discrete spot. If TLC is being used to prepare pure samples ("preparative TLC"), the sample is applied as a large streak made by overlapping spots straight across a large (20cm square) plate to within 1cm of the edges, or by using a commercially available "Streaker" (Applied Science, Inc., State College, Penn.). The sample load may vary considerably and good results still be obtained. One may plate as little as 0.5μg of a single component (on a 0.25mm plate) and still be able to detect it. A load of 1500μg on a 0.25mm plate (to be developed in 2 dimensions) is not uncommon, although loads of 250 to 500μg are more usual. With preparative TLC plates (layer thicknesses of 0.5 to 1mm), several milligrams may be plated.

As a precaution against oxidation, samples should be plated under a flow of nitrogen. Application chambers through which nitrogen can be passed are available commercially; however, one can easily be made from Plexiglas (Fig. 3). A box about 25cm square and 3cm deep is made with an inlet and outlet for a nitrogen line. A narrow (1cm or less) slot is made in the lid to coincide with a line about 1cm from the bottom of a plate placed in the chamber. A sliding cover is fitted over the slot so that the plate can gradually be covered as the sample is applied. A good flow of nitrogen is allowed to pass through the chamber during plating.



Chamber for applying samples to TLC plates under a flow of nitrogen. (Fig. 3)

Chromatograms are developed in glass chambers, in wide-mouth Erlenmeyer flasks (for small plates), or in special "S-chambers." The most commonly used are glass chambers 10¾" × 2¾" × 10½" (Brinkmann Instruments, Inc., 115 Cutter Mill Road, Great Neck, Long Island, N.Y.; Analabs, Inc., P.O. Box 1501, North Haven, Conn. 06473; and other suppliers). The sides of the chambers should be lined with Whatman No. 3 paper; just before use, 250ml of the solvent to be used for developing the chromatogram should be placed in the chamber, tilting the chamber back and forth to saturate the paper with the solvent. A heavy glass lid is used without a grease seal.

For routine checking of column chromatography fractions, it is often convenient to have a series of 1-liter wide-mouth Erlenmeyer flasks covered with one-half of a Petri dish. Each flask will accommodate two 4cm × 20cm plates. S-chambers are commercially available vessels which sandwich plates within a narrow space, thus providing an area uniformly and highly saturated with the solvent vapor. This type of chamber is frequently used with the commercially available precoated plates ("Prekotes," Applied Science, Inc.) or adsorbent coated plastic sheets (Eastman Kodak). The S-chambers generally give the most consistent results.

Detection of Lipids on Chromatograms

Treatment of developed plates with a variety of reagents allows visualization of separated components and possible identification of specific lipid functional groups. When combined with the chromatography of standards developed under the same conditions, detection of specific groups in most cases allows the identification of individual lipids.

There are three general ways in which separated components may be visualized. These methods show the position of the spots but give little or no further information about the compound. These procedures are as follows:

1. Spray[1] with a fluorescent reagent such as rhodamine or 2,2-dichlorofluorescein and view under ultraviolet light. *Protective glasses must be worn when using UV.* The rhodamine reagent is either Rhodamine 6G or B, 0.05 percent by weight in 96 percent ethanol, and the dichlorofluorescein is 0.2 percent by weight in 90 percent ethanol. The plate must be viewed while still wet with the spray reagent.

2. Spray with a solution of 55 percent by weight sulfuric acid and 0.6 percent by weight potassium dichromate and char in an oven at 120°C.

3. Expose to iodine vapor in a closed chamber.

Method 2, the charring procedure, is fairly specific for lipids, but carbohydrates also react. As little as 0.5μg of a compound may be detected in this way. However, there is the disadvantage that the lipids are destroyed and cannot be recovered for further investigations. Method 1, especially the dichlorofluorescein, is the method of choice if the lipids are to be recovered for further analysis. Unfortunately, visualization of small amounts of phospholipids is difficult with this method. Method 3 is excellent in most cases but depends upon the presence of unsaturated lipids, the reaction involved being that of iodine with double bonds. Some saturated nitrogenous lipids (for example, phosphatidyl serine) will take up iodine, but most other saturated lipids will not. Since most natural lipids contain some unsaturated moieties, this procedure can be widely used. Iodine vapor should not be used when lipids are to be recovered for fatty acid analysis since some iodine may remain associated with double bonds and lead to erroneous results.

Sprays that can be used to detect the presence of specific elements or groups and, therefore, the presence of lipid classes are listed below.

Acid-molybdate spray for detection of phosphorus-containing lipids. This spray consists of 5ml of 60 percent perchloric acid, 10ml 1N hydrochloric acid, and 25ml of 4 percent w/v ammonium molybdate. Make up to 100ml with distilled water. Spray and heat in oven at 110°C. Blue spots on gray background are given by phosphorus-containing lipids. Sensitivity is about 1μg.

Ninhydrin spray for detection of lipids containing free amino groups. A positive reaction to this spray is obtained with phosphatidyl ethanolamine and serine, their lyso derivatives, and any other lipid with a primary amine group.

The spray is a 0.2 percent solution of ninhydrin (1,2,3-indantrione-hydrate) in n-butanol-10 percent aqueous acetic acid 95:5 v/v. Spray and heat in oven at 110°C. Purple spots indicate the presence of free

[1] A number of spraying devices are available commercially and prepacked sprays can be purchased. One useful spraying aid is the Sprayon-jet pack power unit (Sprayon Products, Inc., Cleveland, Ohio). *Note: Use all sprays in a hood.*

amino groups. Phosphatidyl serine does not always react well unless some acetic acid (from a developing solvent) remains on the plate; hence, the plate should be sprayed soon after development.

Dragendorff reagent for choline-containing lipids. Positive results are given by phosphatidyl choline, its lyso derivative, sphingomyelin, and related compounds.

A solution of 8.0g bismuth subnitrate in 20 to 30ml 30 percent nitric acid (density 1.18) is added slowly, with stirring, to a solution of 28g potassium iodide and 1ml 6N hydrochloric acid in approximately 5ml water. The dark precipitate obtained will redissolve giving an orange-red solution. Cool this to 5°C and filter. Make up to 100ml with distilled water. This is the stock solution. If stored in a dark bottle and refrigerated, the stock is stable for 1 to 3 months. To make the spray reagent, combine the following in the given order: 20ml water, 5ml 6N hydrochloric acid, 2ml stock solution, and 5ml 6N sodium hydroxide. If the precipitate of bismuth hydroxide does not redissolve even on shaking, add a few drops of 6N hydrochloric acid. The spray solution is stable for 10 to 14 days in a refrigerator. Spray heavily. Positive is a dark orange on a yellow background. Sensitivity is 10μg.

2,4-dinitrophenylhydrazine spray for free aldehyde and keto groups. A positive reaction is given by plasmalogens, but the spray is not very sensitive. The Schiff reagent spray is better (see below).

Spray heavily with a 0.5 percent solution of 2,4-dinitrophenlhydrazine in 2N hydrochloric acid. Yellow or orange spots are positive.

Schiff reagent spray for plasmalogens. Schiff reagent is prepared by dissolving 1g basic fuchsin and 10g sodium metabisulfite in 10ml concentrated hydrochloric acid and 100ml water. This solution is treated for 1 hour with charcoal, filtered, and made up to 500ml with distilled water. This stock should be colorless. It is stable in a refrigerator for several months. The spray reagent consists of 250ml 0.05 percent sodium metabisulfite, 2.5ml 0.05 M mercuric chloride (1.35g in 100ml water), and 2.5ml stock Schiff reagent. Spray heavily and allow several minutes for the reaction. Mauve spots are positive.

Diphenylamine spray for glycolipids. Positive results with this spray are given by all glycolipids, such as the cerebrosides.

The spray consists of 20ml of a 10 percent (by weight) ethanolic solution of diphenylamine, 100ml concentrated hydrochloric acid, and 80ml glacial acetic acid. Spray and heat for 5 to 10 minutes at 100 to 120°C. Blue-gray spots on a light gray background are positive. Sensitivity is within the range of 4.0 to 10.0μg.

α-naphthol spray for glycolipids. This spray gives positive results with sterols and steroid conjugates, as well as with glycolipids.

The spray consists of 10.5ml of a 15 percent (by weight) solution of α-naphthol in ethanol, 6.5ml concentrated sulfuric acid, 50.5ml

ethanol and 4ml water. Heat for 3 to 6 minutes at 100 to 110°C. Spots of various colors are positive.

Resorcinol spray for gangliosides. Two grams of resorcinol are dissolved in 100ml distilled water. Ten ml of this solution are added to 80ml of concentrated hydrochloric acid containing 0.25ml of 0.1M copper sulfate, and the resulting solution is made up to 100ml with distilled water. This spray reagent is stable for about one week in the refrigerator. Spray and heat in oven at 110 to 120°C in closed jar. Gangliosides give violet-blue reaction; other glycolipids go yellow.

Other spraying methods. Many other sprays for specific groups can be applied to TLC plates. Generally any spray reagent used in paper chromatography can be applied with little or no modification to TLC plates. The TLC plate has an advantage over the paper chromatogram in that corrosive sprays can be used. The very corrosive procedure of spraying with the sulfuric acid-chromate solution has the advantage that observation of the spots during charring may provide additional information. Sterols, such as cholesterol, and various bile acids undergo brilliant color changes, from green through red-purple, before blackening.

As with paper chromatograms, if radioactively labeled lipids are used, spots may be detected by exposing the plates to X-ray film. In these cases a permanent record is obtainable in the form of an X-ray print. Otherwise, results can be documented by copying the patterns into a notebook or by taking photographs with a Polaroid camera.

Whatever visualization and detection techniques are employed, it is absolutely essential that standard lipids are run under the same conditions. Runs should also be made using more than one solvent system before identifications are made. Identification should never be made on the basis of R_F values[2] alone since separations may vary with differences in humidity, batch of adsorbent, etc. If the sample contains rare lipids or is derived from a source not previously examined by other investigators, any unusual lipids should be assigned an identity only when additional aids have been employed. In such cases it is necessary to isolate enough of the component in pure form to be examined by mass spectrometry, infrared spectroscopy, and other analytical aids.

Separation of Neutral Lipids

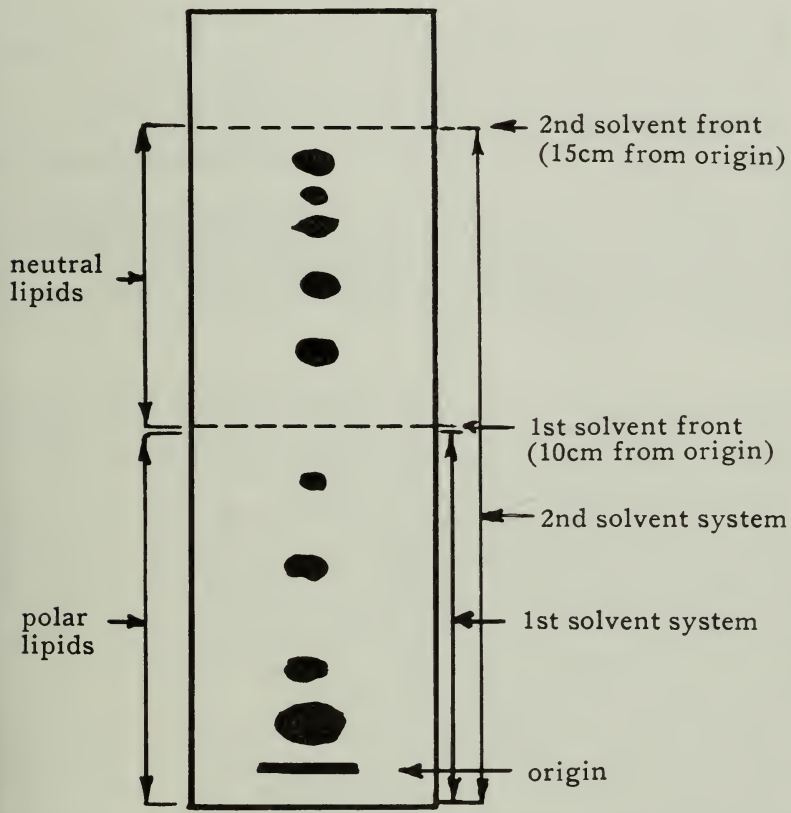
The choice of solvent systems for the development of chromatograms is quite wide and depends on the adsorbent used, the temperature and humidity conditions in the laboratory, and the complexity of the mixture to be resolved.

In general, mixtures of low boiling petroleum hydrocarbons containing various amounts of diethyl ether and benzene will separate

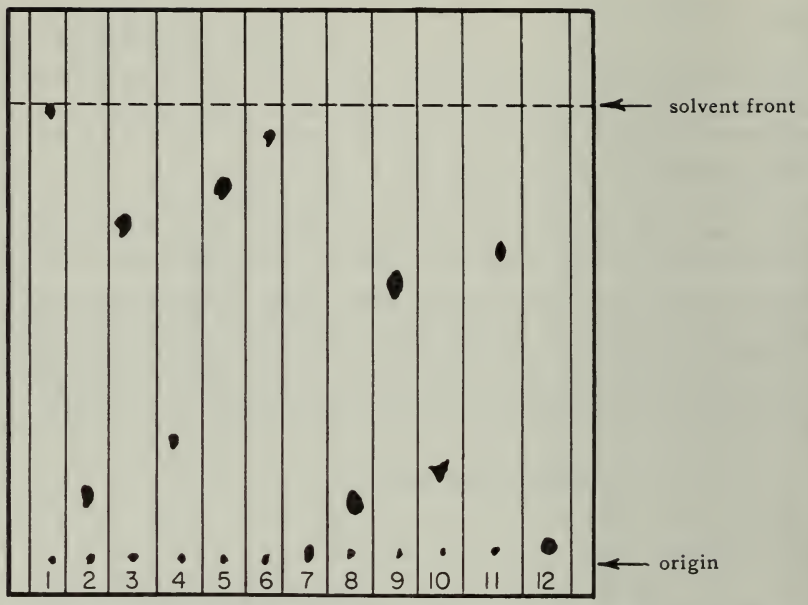
[2] R_F value $= \frac{\text{distance from origin to component spot}}{\text{distance from origin to solvent front}}$

most neutral lipid mixtures. Neutral lipids are readily separated from the polar in a number of ways. In many cases, development of the chromatogram with a more polar solvent mixture such chloroform-methanol-water 65:25:4 (all mixtures are given by volume) will move the neutral lipids to or near the solvent front, whereas the polar lipids will lag behind to varying degrees.

If free fatty acids, cerebrosides, and cardiolipin are present, however, there will be fraction overlap. In these cases the technique of multiple development is useful. With this procedure, the plate is developed with the chloroform-methanol-water system to a height of about 10cm, instead of the usual 15cm employed with average plates. The plate is then dried and redeveloped in the same direction with a less polar solvent, such as hexane-ether 4:1, to a height of 15cm (Fig. 4). This solvent will hardly move the polar lipids, but will redistribute the



Separation of polar from neutral lipids by multiple development technique. (1) Developing solvent allowed to rise to height of 10cm (chloroform-methanol-water 65:25:4). (2) Developing solvent developed to height of 15cm (hexane-ether 4:1). Adsorbent, Silica Gel G. (Fig. 4)



Separation of some neutral lipids. Solvent system hexane-diethyl ether-glacial acetic acid 90:10:1.

(1) 9-octadecene, (2) oleyl alcohol, (3) oleylaldehyde, (4) oleic acid, (5) methyl oleate, (6) cholesterol oleate, (7) monolein, (8) diolein, (9) triolein, (10) cholesterol, (11) tristearin, (12) phosphatidyl choline. Note that the polar lipid (12) and monolein (7) do not move from the origin. Adsorbent, Silica Gel G. (Fig. 5)

neutral lipids in the 5cm band above the polar lipids. It should be noted here that if the lipids are to be recovered for further analysis, especially for fatty acid and fatty alcohol analyses, the plates must be dried in a box through which there is a flow of nitrogen.

Figure 5 shows the separation of some neutral lipids. The solvent system is hexane-diethyl ether-glacial acetic acid 90:10:1. The glacial acetic acid in the system prevents tailing of the more acidic components. Similar separations can be obtained using a system without the acid and with differing proportions of hexane-ether. The more polar the system (that is, the more diethyl ether, or acid, or both, it contains), the further it will move the relatively more polar materials.

Isomeric mono- and diglycerides can be separated by TLC. As we previously noted (p. 36), 1- and 2-monoglycerides can be separated on columns impregnated with boric acid. Similarly, these isomers may be separated on Silica Gel G TLC plates which have been impregnated with ammonium borate. The solvent system is chloroform-acetone 96:4. The 1,2- and the 1,3-diglycerides may be separated on Silica Gel G by

using petroleum hydrocarbon (boiling range 40 to 60°C)-diethyl ether 70:30.

Numerous other systems are available for special separations of neutral lipid mixtures. Many of the earlier ones were reviewed by Mangold and Malins (*4*), and more recently by Renkonen and Varo (*5*).

Argentation Thin-Layer Chromatography

Fatty acids, their methyl esters, cholesterol esters, and glycerides can be separated according to degree of unsaturation by TLC on silica gel impregnated with silver nitrate. The *cis* and *trans* isomers of some fatty acids can also be separated in this way.

Silver nitrate plates can be made in several ways. Some investigators prepare plates by spraying silica gel plates with a 10 percent solution of silver nitrate in aqueous ethanol. "Prekotes" or other commercially available plates can be impregnated with silver nitrate by dipping the plates into a 10 to 12 percent solution of silver nitrate. The most reliable procedure, however, is to prepare the silica gel slurry in silver nitrate solution rather than water and to spread the plates in the usual manner. The following formula will generally give satisfactory plates, although it may be necessary to adjust to the prevailing humidity conditions.

Weigh 25g silver nitrate and dissolve in 200ml distilled water. Weigh 60g silica gel and prepare a slurry using 136ml of the silver nitrate solution. Make sure that the slurry is very thoroughly mixed by using a Waring Blendor or by shaking vigorously in a stoppered flask for about 10 minutes. Prepare plates in the usual way; dry and store plates in a dark place.

These plates can be spotted in dull light; however, they should be developed in a dark jar or the developing chambers should be placed in a dark place. Depending upon the separations desired, hexane-diethyl ether in varying proportions will be found suitable in most cases. Figure 6 illustrates the separation of several cholesterol esters on silver nitrate impregnated Silica Gel G. The plates were developed with hexane-ether 93:7 for monoenoate separation and 93:17 for dienoates. To separate the more unsaturated compounds (trienoates, tetraenoates, etc.), increasing quantities of ether in hexane must be used. For example, a mixture of saturated, monoene, diene, triene, tetraene-pentaene-hexaene (polyene fraction) of fatty acid methyl esters can be separated into 5 bands by using 60:40 hexane-ether, although there may be slight overlap. Lipids can be recovered from the scraped off silica gel by extraction with moist diethyl ether. Rechromatography on silver nitrate impregnated plates will yield purified lipid fractions.

Solvent systems similar to those described above can be used to separate various glyceride mixtures on the basis of unsaturation. Each

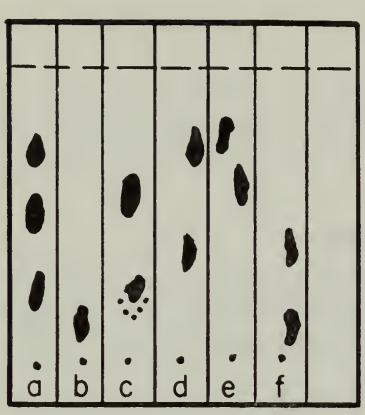


Plate 1: monoenoates

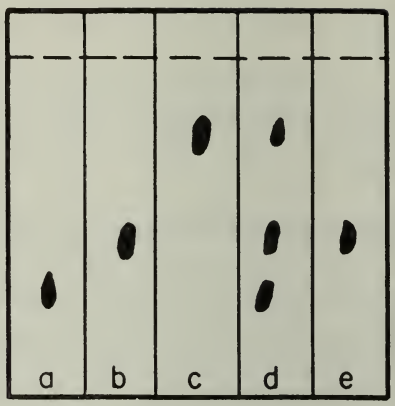


Plate 2: dienoates

Thin-layer chromatography of cholesterol esters on Silica Gel G impregnated with silver nitrate. Plate 1: (a) mixture of cholesterol palmitate, elaidate, oleate; (b) stearolate [$CH_3(CH_2)_7C \equiv C(CH_2)_7COOH$ is stearolic acid]; (c) mixture of elaidate, oleate, stearolate; (d) *trans* and *cis* vaccenate; (e) *trans* and *cis* erucate; (f) *trans* and *cis* petroselenate. Plate 2: (a) linoleate; (b) 9-*cis*-12-*trans* octadecadienoate; (c) linelaidate; (d) mixture of a, b, and c; (e) 9-*trans*-12-*cis* octadecadienoate. (After Sgoutas, *13*.) (Fig. 6)

investigator will, however, find some trial runs necessary in order to ascertain the particular solvent systems suited to his conditions.

Separation of Phospholipids and Glycolipids

Numerous solvent systems for one-dimensional TLC of phospholipids and glycolipids have been reported. Among those that give satisfactory separations of mixtures containing most of the common phospholipids and cerebrosides are: (a) chloroform-methanol-28 percent aqueous ammonia 75:25:4 (on Silica Gel G); (b) chloroform-propionic acid-n-propanol-water 10:10:10:4 or 2:2:3:1 (on Silica Gel G plates prepared in 5 percent ammonium nitrate); (c) chloroform-methanol-acetic acid-water 25:15:4:2 (on Silica Gel G plain prepared in 0.001M sodium carbonate).

For routine checking of column chromatography fractions and for many analyses, unidimensional systems are suitable. If, however, it is desired to separate all the phospholipids and glycolipids of a multicomponent mixture or to characterize a lipid mixture from a new source, then two-dimensional TLC must be used. Furthermore, it is highly desirable that the number and identity of components in a complex mixture be established with more than one set of developing solvents. Several such systems have been developed by Rouser *et al.* (*2*).

In two-dimensional TLC the sample is spotted in one corner of a 20cm × 20cm plate and developed in one direction with one solvent system; then, after removal of the first solvent, the plate is turned through 90° and developed in the second direction with solvent 2. The adsorbents used may be any commonly used TLC adsorbent. The chromatogram maps shown in Figure 7 (p. 54) illustrate separations obtained by using pairs of solvent systems.

In the separations shown in Figure 7, the gangliosides are present as one spot. However, gangliosides can be separated into mono-, di-, tri-, and tetrasialogangliosides by using several solvent systems. The system described by Kuhn and Wiegandt (*6*) gives good separation of the major gangliosides. The solvent system is propanol-water 7:3 (on Silica Gel G). Chloroform-methanol-water 60:35:8 (on Silica Gel G) also gives good separation of gangliosides (*7*). Leedeen (*8*) has described a multiple development system that gives good resolution of major gangliosides; double length (40cm) plates and two sucessive runs of chloroform-methanol-2.5N ammonia 60:40:9 are used.

Quantitative Thin-Layer Chromatography

There are several possible approaches to quantitative analysis by TLC. These approaches can be divided into four main groups.

1. Direct analysis on the TLC plate.
   a. Charring under standard conditions and subjecting the plate to photodensitometry.
   b. Direct radio scanning (when radioactively labeled lipids are being handled).
   c. Neutron activation analysis.
2. Gravimetric analysis by recovery of the lipids from each spot.
3. Analysis for specific elements or functional groups on lipids extracted from the adsorbent.
4. Analysis for specific elements or functional groups on lipid plus adsorbent.

In the first group, charring followed by densitometry has been used successfully by a number of investigators. This method is, however, often difficult and tedious to standardize. Standard lipids must be charred and separate curves prepared for the densitometry of each. The size of spots for each lipid should be fairly consistent from day to day, which is often difficult to achieve. Moreover, charring should ideally convert the lipids quantitatively to carbon, but this is not easily achieved in practice. Other detection methods besides charring have been successful. Such methods are usually specific for lipids containing one specific group; for example, staining with chlorox-benzidine reagent is specific for amide groups. Sphingolipids have been analyzed by densitometry after having been stained by this method (*9*).

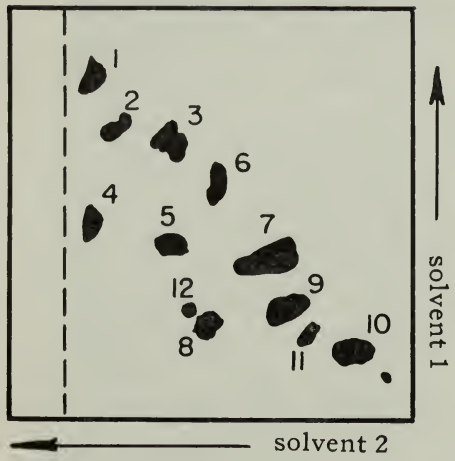


Plate 1

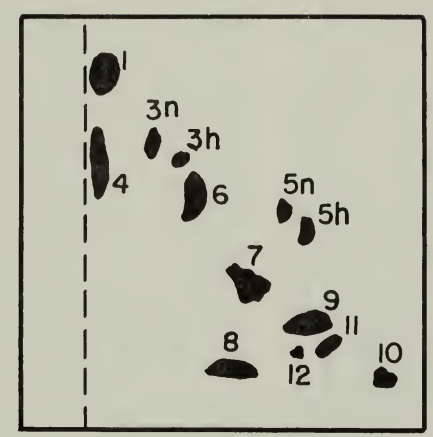


Plate 2

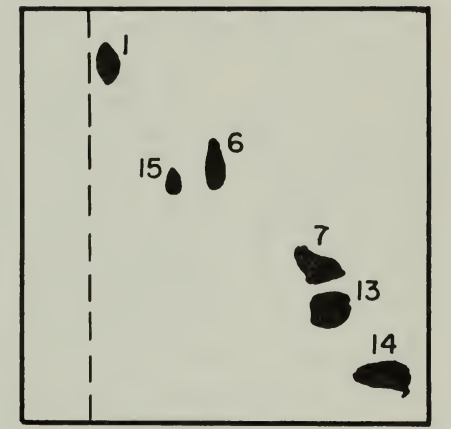


Plate 3

Two-dimensional thin-layer chromatography. Adsorbent, Silica Gel G + 10 percent magnesium silicate. Plate 1: solvent 1 = chloroform-methanol-water 65:25:4; solvent 2 = n-butanol-glacial acetic acid-water 60:20:20. Plates 2 and 3: solvent 1 = chloroform-methanol-28 percent aqueous ammonia 65:35:5; solvent 2 = chloroform-acetone-methanol-glacial acetic acid-water 10:4:2:2:1. (Fig. 7)

1. Neutral lipids (such as sterols, triglycerides, etc.)
2. Cardiolipin
3. Cerebrosides with normal (n) and hydroxy (h) fatty acids
4. Free fatty acids
5. Sulfatides with normal (n) and hydroxy (h) fatty acids
6. Phosphatidyl ethanolamine
7. Phosphatidyl choline
8. Phosphatidyl serine
9. Sphingomyelin
10. Gangliosides
11. Lysophosphatidyl choline
12. Phosphatidyl inositol
13. Ceramide aminoethylphosphonate
14. Ceramide polyhexosides
15. Diphosphatidyl glycerol

Radio scanning of TLC plates, using a proportional gas-flow counter tube, is generally successful but is of limited value.

Neutron activation analysis has been tried and promises to be of great value in the analysis of very small quantities of lipids. This approach awaits further development.

Gravimetric analysis is not generally a reliable approach. It is often adequate on a preparative TLC scale when large quantities are being chromatographed and recovered. At lower levels, however, it is often difficult to recover lipids completely from the adsorbent, particularly polar lipids. It is not recommended as an analytical procedure.

The third approach suffers the same disadvantage as gravimetric analysis in that complete or consistent recovery of lipids from the adsorbent is frequently difficult and tedious.

The fourth approach is less cumbersome than other methods and, in general, is the best procedure. It is essential, however, that precautions are taken against interference with the analysis by the adsorbent itself, by impurities in it, or by reagents used for localization of the spots. Exposing plates to iodine vapor and outlining spots with a needle is ideal in many cases. The iodine resublimes fairly rapidly and the spots can then be removed either by scraping or aspirating off. This method cannot be used if the lipids are being removed for analysis of their fatty acid moieties, since some iodine may remain irreversibly added to double bonds. In such cases, it is best to localize spots by spraying with one of the fluorescent dyes like 2,7-dichlorofluorescein.

After the spots are removed and put into suitable containers, many analyses can be carried out directly on the adsorbent plus lipid. For example, phosphorus analyses for the relative proportions of phospholipids can be carried out in the presence of adsorbent directly after digestion of the lipid. Detailed instructions for determination of organic phosphorus and other analytical procedures are given in Chapter 6. Procedures for the analysis of fatty acids and fatty aldehydes of lipids recovered from TLC plates are given in the chapter on gas-liquid chromatography.

A scheme for analysis of phosphoglycerides. Lipids from natural sources are complicated mixtures of different molecular species. A triglyceride preparation that contains only two different fatty acids could actually be a composition of six different species. Thus, with fatty acids A and B, we can have triglycerides AAA, BBB, AAB, BBA, ABA, and BAB. Similarly, with a phosphoglyceride containing only two fatty acids, we could have four species: AA, BB, AB, BA. With increasing frequency, answers to the role in biological systems demand that we know the pattern of the existing molecular species.

In the case of glycerides, there is already a vast amount of knowledge about determining structural patterns, and references to pertinent methods and results are given in Suggested Further Readings. In the

case of polar lipids, however, only in the last few years has progress been made in the analysis of molecular species. Renkonen (*10*) demonstrated that much could be learned about the molecular species of many natural phosphatides by examining the diglyceride parts of these molecules by thin-layer chromatography. Kuksis and Marai (*11*) extended this approach to include examination of the derived diglyceride acetates by gas-liquid chromatography. They first applied this technique to the determination of the complete structure of egg yolk lecithins. More recently Kuksis *et al.* (*12*) have shown that the method can be applied successfully to the determination of the molecular species of the lecithins from rat heart, kidney, and plasma. The outline of their procedure (summarized in Fig. 8) is as follows:

1. The phosphoglycerides to be studied are isolated and purified by column or preparative thin-layer chromatography. Aliquots of the isolated pure phosphoglycerides are taken for immediate transmethylation. The methyl esters of the fatty acids thus obtained are analyzed by gas-liquid chromatography (see Chapter 5 for details of GLC).

2. Separate aliquots of the pure phospholipids are converted directly to the diglycerides by dropping the removed silica gel bands into a buffered phospholipase C solution (*10*). The incubations are continued for 30 to 60 minutes at 28 to 30°C under a layer of diethyl ether.

3. At the end of digestion the phases are separated and further extractions with ether are made. The combined solvents are evaporated.

4. The diglycerides obtained are purified by TLC on Silica Gel H (Merck & Co.) using petroleum ether-diethyl ether-formic acid 120:40:3 v/v/v as the developing solvent.

5. The diglycerides are recovered from the scraped-off bands (using 2,7-dichlorofluorescein for location) by elution with chloroform.

6. The diglycerides are dissolved in pyridine and converted to the diglyceride acetates by treatment with acetic anhydride at room temperature for 12 hours. If the original lipid sample is 400 to 500mg, the diglycerides obtained should be dissolved in about 0.1ml pyridine and treated with 0.25 to 0.50ml acetic anhydride.

7. The diglyceride acetates are separated according to degree of unsaturation by TLC on 0.25mm thick Silica Gel G plates (20cm × 20cm) containing 20 percent silver nitrate. About 10 to 15mg of material is applied as a band and the plate is developed twice in the same direction with a solution of 0.7 or 0.8 percent methanol in chloroform. The bands are located using the fluorescein reagent. The individual bands are recovered by eluting with chloroform-methanol 9:1 v/v to which 5 percent water has been added.

8. The eluates are reduced to dryness under nitrogen and subjected to gas-liquid chromatographic analysis.

Diagram of analysis scheme for phosphoglycerides. (Fig. 8)

9. The mixture of diglyceride acetates (from step 6) is analyzed by gas-liquid chromatography in order to obtain the overall molecular weight distribution.

10. Further information is obtained by subjecting the diglyceride acetates to hydrolysis by pancreatic lipase, which selectively removes the fatty acid in the $\alpha(1)$-position. The free fatty acids and monoglycerides obtained are removed separately after thin-layer chromatography as described for the diglycerides (step 7).

11. The positional distribution of the fatty acids in the *original* mixture is determined by hydrolysis with phospholipase A, which removes the fatty acids in the $\beta(2)$-position; the fatty acids are then converted to their methyl esters and analyzed by gas liquid chromatography. This allows for verification of the positional distribution of the fatty acids derived from the lipase digestion.

The two enzymic incubations in this procedure are carried out as follows:

The lipase digestion of the diglyceride acetates. Fifty to 100mg of sample is added (in a 5ml screw cap vial) to a predetermined weight of pancreatin (Steapsin, Nutritional Biochemical Corp.). The amount of enzyme chosen is that which hydrolyses 50 percent of a given weight of diglyceride acetate in 1 to 2 minutes. One ml of 1M trishydroxymethylamino methane (pH 8.0), 0.1ml 22 percent calcium chloride, and 0.25ml 0.1 percent bile salts solution are added to sample plus pancreatin. The vial is warmed in a water bath at 40°C for 1 minute. The cap is then screwed on tightly and the vial is shaken for the time required; for 50 percent hydrolysis, this is usually 1 to 2 minutes. At the end of reaction the contents are acidified with 6 N HCL and extracted with ether.

The phospholipase A digestion of the phosphoglyceride. From each sample, 150 to 200mg of phosphoglyceride is dissolved in 100ml diethyl ether and treated with 1ml of 0.1 percent rattlesnake venom (*Crotalus adamenteus*) in 0.005M $CaCl_2$ solution. The solution is allowed to stand overnight, after which the precipitate is removed by centrifugation and washed once with diethyl ether. The ether phase should be free of phosphorus (it should contain only free fatty acid) if the reaction is complete.

This scheme, therefore, supplies the following information:

1. The total fatty acid composition of the phosphoglyceride (step A in Fig. 8).

2. The overall molecular weight distribution of the diglyceride acetates derived from the phosphoglyceride (steps B, C, D).

3. The overall molecular weight distribution and proportional contribution of the diglyceride acetates according to their degree of unsaturation (steps B, C, E).

4. The overall composition and positional placement of the fatty acids in the derived diglyceride acetates (steps B, C, G, H).

5. The fatty acid composition of position 2 in the original phosphoglyceride (steps I & J), a verification of the information derived in 4.

From the above information it is, therefore, possible to deduce the position of the fatty acids in the phosphoglyceride and to describe the molecular species present. The method allows for cross-checking in that the total fatty acids composition of the original phospholipid (under 5 above) should be the same as that of the derived diglyceride acetates (under 4 above).

The conditions suitable for the GLC analysis steps in the above scheme are to be found in the next chapter (see pp. 76-78, 82).

PAPER CHROMATOGRAPHY

Early attempts to apply paper chromatography to the resolution of naturally occurring lipid mixtures were only partially successful. It was not until chromatography on impregnated papers was introduced that more successful separations were made. Papers have been treated in various ways, including acetylation and impregnation with tetralin and formalin. The most successful and widely used procedure is chromatography on paper impregnated with silicic acid, a technique developed mainly by Marinetti (*14*) and his co-workers. This approach enjoyed wide popularity for some time until the development of TLC procedures, which are more rapid and more versatile than paper chromatography and allow a wider range of sample loading. Paper chromatography is, however, still popular in a number of laboratories for some special purposes. A number of reviews give details of paper chromatographic procedures (*14, 15, 16*).

One special use of paper chromatography in lipid analysis should be mentioned here, the method of phosphatide analysis introduced by Dawson in 1954. Very mild hydrolysis is used to deacylate phosphatides, after which the water soluble phosphorus-containing compounds are analyzed by paper chromatography. Again, after the introduction of TLC this method declined in popularity. It does, however, offer superior resolution of the acidic phosphatides. If a detailed analysis of previously uncharacterized lipids is being undertaken, this method provides a valuable adjunct to TLC and column procedures for the analysis of intact lipids. Dawson (*17*) has recently reviewed this procedure in detail and discusses those areas in which it offers special advantages.

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V. Gas-Liquid Chromatography

So far we have considered chromatographic techniques in which the moving phase was a liquid and the stationary phase a solid. In gas-liquid, or gas-liquid partition, chromatography (GLC), the moving phase is a gas and the stationary phase is a liquid. The liquid is spread over an inert solid support, usually a flux-calcined, diatomaceous earth of fairly small particles. Separation takes place in a series of partitions whereby the sample goes into solution in the liquid phase and is subsequently revaporized. The length of time it takes a component to emerge from a GLC column depends upon the affinity it has for the liquid phase and upon its boiling point. Thus components are separated because of differences in their affinity for the adsorbent and differences in their boiling points.

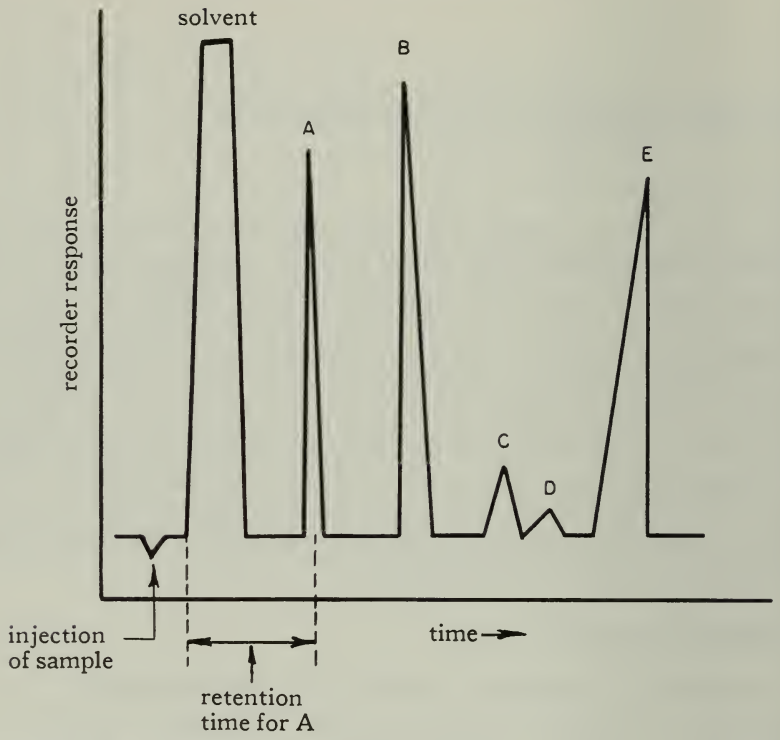
INSTRUMENTATION

A typical GLC apparatus consists of the following units:

carrier gas (moving phase)	→ injector system	→ column packed with solid support coated with liquid phase; column enclosed in oven	→ detector system	→ signal recorder

The various components of an injected sample separate into discrete bands in the gas flow and move through the column at different velocities. As they emerge, they pass through a detector system and a signal is sent to a millivolt recorder. The record so obtained is termed the chromatogram. A typical chromatogram is shown in Figure 9.

The time lapse measured between sample injection and the apex of a component peak is termed the retention time. Frequently, a small positive or negative recorder response at the point of injection makes a suitable start for measurement. If this response is not obtained, measurements may be made starting at the apex of the solvent peak. The distance may be measured in any units (cm., inches); the retention time is then calculated from this measurement and the speed of the recorder chart. Before discussing the routines for making qualitative and quantitative analyses, each unit of the typical GLC instrument will be considered in a little more detail.



A typical gas chromatogram. **(Fig. 9)**

Carrier gases. The selection of carrier gas is dependent upon the type of detector, as shown here:

Detector	Carrier gas
Flame ionization	Nitrogen, helium, argon
Thermal conductivity	Hydrogen, helium
β-ionization	Argon
Electron capture	Argon

The carrier gas must be pure, inert, and dry. It is best to dry the gas by passing it through a molecular sieve, which can be reactivated periodically by heating at approximately 350°C for four hours with the carrier gas flowing through it. Impure or wet carrier gas will lead to such problems as the appearance of "ghost" peaks, decreased column life, baseline drift, and noise.

The carrier gas should pass through a flow regulator, as carrier gas flow rates must be adjusted to achieve optimum efficiency. Optimum flow rates will vary from column to column.

Injection system. The usual injection system consists of a heated injection port with a self-sealing rubber or silicone septum through which the sample can be introduced into the gas stream with a microliter syringe. Most injection ports allow for projection of the column into the injection area so that on-column injection is possible.

The injection port temperature must be controlled and must be high enough to vaporize samples. The injection system should be leakproof and must be so designed as to prevent back flow into the carrier gas inlet. The velocity of gas flow through the injection port must sweep the vaporized sample rapidly into the rest of the system.

Column technology. This is the most important part of the chromatograph. Problems can often be traced to a faulty column. The column consists of three parts: the container, which may be metal (copper, stainless steel) or glass; the solid support; and the stationary phase.

For some analyses, including the analysis of sterols, a glass column and injection port are essential, since metal, especially copper, may catalyze sample breakdown. In general, however, stainless steel is satisfactory.

The purpose of the solid support is to hold the liquid phase. The support can, however, influence events occurring within the column by adsorbing components at active sites or by catalyzing reactions due to trace metal impurities. The former problem is alleviated by treating the support with a silylating reagent, such as bis-trimethylsilylacetamide (BSA). Acid washing will remove metals.

The choice of column, solid support, and liquid phase varies with the separations being attempted. The catalogs of all leading suppliers generally recommend specific packings for various types of separations. In the sections that follow, suggestions for column packings are included in the discussions of the various separations.

Length of column may vary from a few inches to about one mile. The latter extreme is the case for capillary or Golay columns, in which the support and liquid phase are merely coated on the inside of capillary tubing. Such columns are especially useful in some tricky situations involving geometric isomer separations. For general use, columns are 18 inches to 6 feet long and U-shaped, straight, or coiled, depending on the instruments.

The temperatures at which columns are used depends on the compounds being analyzed but above all on the stability of the stationary phase. The maximum temperature at which various phases are stable is reported by suppliers. This is a most important consideration since excessive bleed of liquid phase from the column or its breakdown will lead to problems, including fouling of the detector system. The percentage of liquid phase used varies from as little as 1 percent to as much as 30 percent; most analyses, however, are achieved with concentrations of 1 to 10 percent. Before use, a new column is always

"bled out" (usually overnight or for 24 hours) to rid the column support of excess phase. This is generally done with the column *detached* from the detector.

Columns may be operated isothermally for analysis at a predetermined optimum temperature, or the temperature may be programmed to rise from a given lower temperature to a given maximum temperature during each run. The rate of rise of temperature may vary from 3 to 18°C per minute. Temperature programming is especially useful when samples with a wide range of boiling points are being analyzed or when samples contain components that elute over a long period of time. Programming is not merely a time saver, however; it also improves the general symmetry of the recorded peaks. As we shall see, this is a big advantage in quantitative analysis. The longer a component remains on a column, the broader its band becomes as it diffuses during passage through the column. The result is loss of peak response, since small amounts of the diffused band (with and against the direction of carrier gas flow) are not detected. Instead, the peak may appear as a long hump on the baseline. Temperature programming reduces the length of time that high-boiling and slow-eluting components remain on the column, and the decrease in diffusion leads to sharp, well-defined peaks.

Detector systems. The separation achieved by the column must be translated into an electrical signal which can be fed into a recorder and used for qualitative and quantitative analysis. The translation is done by a detector. The "perfect detector," one which responds linearly and identically to any amount of any type of compound, does not exist. Each detector has a finite range within which response changes linearly with the quantity of components. This is called the linear dynamic range (LDR) of the detector. If the detector is used outside its LDR, the peak shapes become distorted and reproducibility decreases. Detector responses also depend on the functional groups on the component being analyzed. An equal number of moles of a hydrocarbon and a fatty acid methyl ester will not give the same signal. Some calibration of the detector response is required for every compound being analyzed quantitatively. These and other factors involved in detector technology will be discussed for each type of detector considered.

Of the many kinds of detectors, the most commonly used are the thermal conductivity cells and the ionization-type detectors. *Thermal conductivity* (TC) detectors detect changes of thermal conductivity in the carrier gas when it becomes diluted by components in the sample. The more highly conducting the carrier gas is, the greater will be the thermal conductivity change when a component enters the TC cell. Hydrogen and helium are recommended carrier gases. When the thermal conductivity of the component is less than that of the carrier gas, sensitivity is good but will diminish as the thermal conductivity

approaches that of the carrier gas; response will be a negative peak if the thermal conductivity of the carrier gas is exceeded.

A filament heated by direct current is usually the varying resistance element. Two of these elements or two matched pairs of elements are placed in two streams of carrier gas; they are called the reference and the sensor elements. The temperature of the elements depends on the carrier gas conductivity, its flow rate, and the temperature of the detector block. To decrease noise and increase cell life, filament detector cells must be run as cool as possible, consistent with the boiling points of the sample components.

The typical circuitry for a TC detector is a Wheatstone bridge used as a temperature sensing bridge. When a component dilutes the carrier gas and passes with it over the sensor element, the element heats up and its resistance increases. As a consequence, there is an out-of-balance signal between the sensor element and the reference element (over which pure carrier gas flows). This signal is transposed to a voltage output and fed to the recorder.

The most popular type of TC detector employs the hot wire element. This type of detector has a wide temperature range, does not destroy the component (which can, therefore, be collected and fed into other instruments such as infrared spectrophotometers or mass spectrometers), and has no selectivity so far as any particular type of compound is concerned. If conditions are favorable, a hot wire detector can measure as little as 0.1μg.

The thermistor, another type of varying resistance element, is infrequently used now except for gas analysis. It is very efficient in this, measuring in the parts per million range. Sensitivity decreases above 150°C.

Ionization detectors have more complicated circuitry than TC detectors, are more trouble to maintain, and, since they show selectivity of response, require more detailed calibration and sample handling. They are, however, very sensitive, giving in some cases full-scale recorder deflection in response to 1 picogram (10^{-12} g) of component.

The flame ionization detector (FID) is one of the most popular detectors. It measures the minute current developed when a combustible material in carrier gas enters a hydrogen-air flame. A d.c. potential is applied across the flame by a pair of electrodes. As the component burns, the electrodes collect the charged species and the resulting current is amplified by an electrometer and led to a recorder. Routine use allows analysis of 10 nanograms (1 nanogram = 10^{-9} g), and under favorable conditions 0.1 nanogram can be measured. Since response is a function of the number of carbon atoms and the groups in which they appear, the detector must be standardized and calibrated to handle a specific type of sample. This detector has some degree of selectivity since poorly combustible materials such as carbon tetrachloride give weak signals; the detector is also not suitable for the analysis of many

gases. However, the FID detector is not so easily damaged as the TC detector and is readily cleaned.

Another type of ionization detector is the Argon, Beta Ray, or Alpha Ray type. The usual name is the Argon Ionization Detector, since argon is the commonly used carrier gas for these detectors; Beta Ionization Detector is also frequently used, whether the ionizing source emits β- or α-particles. In this detector, radiation from an isotope source ionizes a small percentage of the carrier gas (argon) atoms, giving rise to an "ionization" or a "standing" current. The isotope sources are strontium 90 or tritium (β-particle emitters) or radium 226 (an α emitter). A few argon atoms receive less than enough energy to be ionized but enough to displace an electron from its normal orbit or shell — about 11.6 electron volts. Such atoms remain in their metastable state until something collides with them. If one collides with a sample component that needs less than 11.6 ev to be ionized, ionization will take place and another electron is set free in the ionization cell. If a polarizing voltage is applied across the cell, these ionized sample molecules will increase the ionization (or "standing") current. Increasing the cell voltage increases the number of metastable atoms as follows: the electrons composing the ionization current accelerate more rapidly; the kinetic energy of the electrons is transferred to the argon atoms during collisions, resulting in more transfers of 11.6 ev; and more metastable atoms are formed which, in turn, ionize more sample molecules. Therefore, raising the cell voltage (and hence the number of metastable argon atoms per unit volume) results in a higher ionization efficiency for sample molecules. As with other detectors, changes in ionization current are fed to a recorder.

Still another type of ionization detector is in common use for certain types of analysis: the Electron Capture (or Attachment) Detector (ECD). Here β-particles generate an atmosphere of electrons and positive ions. The electrons are collected at the cell anode. There is a low voltage on the electrodes when only carrier gas is in the cell. If sample molecules which readily accept electrons enter the cell, the low-energy electrons may attach themselves to the molecules. Negative ions of sample components and positive argon ions combine much faster than electrons and positive ions, hence components which have a high electron affinity reduce the primary ionization current. This detector is much more selective than any we have discussed so far, particularly, for example, towards halogenated compounds. For this reason, the ECD is much used by investigators measuring small quantities of chlorine-containing pesticides. ECD sensitivities are in the picogram range.

QUALITATIVE ANALYSIS

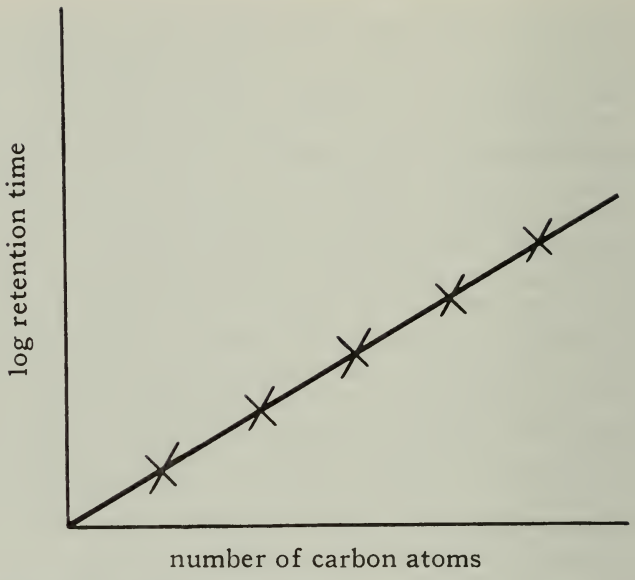
If it is desired to analyze completely unknown samples, GLC is not a suitable technique when used alone; other techniques such as

infrared and mass spectroscopy must also be used. One must, therefore, have some idea of the type of compound being investigated. The only definitive property of a compound measured by GLC is its retention time, and that only to the extent that all conditions are carefully standardized during the chromatographic run. Temperature, gas flows, etc., should be controlled carefully; if temperature is programmed, the rate of rise should be uniform. Also, the retention time should be measured from some reproducible point, for example, as we previously noted, from the solvent peak. The start may be taken as the leading edge of the solvent peak or its apex, whichever is more convenient. With a TC detector the air peak is frequently a suitable starting point. Retention time (RT) itself varies with several instrument parameters, including column temperature, column length, flow rate of carrier gas, type of liquid phase, loading of sample, and weight of column packing. This makes RT not too reliable a figure for correlating data obtained at different times or in different laboratories. A more reliable parameter is relative retention time (RRT), the ratio of the RT of a known standard component to the RT of the sample.

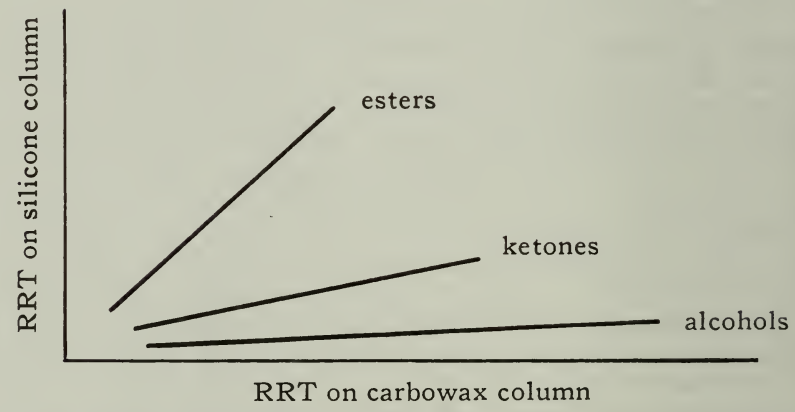
If one has some preliminary information about the unknown obtained by other means (having determined functional groups by spot tests, infrared analysis, etc.), one may proceed with some GLC analysis. For example, suppose that preliminary analysis indicates that the sample is a mixture of aliphatic alcohols: then the number of components, their chain length, etc. can be determined by GLC. Chain lengths are determined by utilizing the fact that for various members within a chemical class (the n-alcohols, the ethers, the ketones, the fatty acids, etc.) log RT varies linearly with molecular weight. Thus, if one chromatographs a series of saturated n-alcohols on a suitable phase (silicone or carbowax), measures retention time, and plots log RT against the number of carbon atoms in the alcohol, a straight line is obtained (Fig. 10). A series of straight line graphs can be obtained from the mono-, di-, and tri-unsaturated species, and so on. Thus, the possession of a series of standards enables one to test the unknown for fit on one of the curves. Ideally, unknowns and standards should be run on the same day and, of course, under standardized conditions of column, flow rate, and temperature.

In addition to determining functional groups by conventional means, it is often possible to use GLC data to find out what type of compound one is dealing with. This requires collecting RRT data for a large number of functional group classes on at least two standard columns, such as silicone and carbowax. Because of the linearity of homolog retention on the two columns, a plot similar to that in Figure 11 can be obtained. Thus, by injecting an unknown on two columns, a compound can be identified that fits several of the prepared standard plots.

The utility of GLC is enhanced when combined with a number of other analytical procedures; in particular, chromatographing an un-



Results of chromatography of homologs. (Fig. 10)



Chromatography of homologs on two columns. (Fig. 11)

known before and after a specific chemical treatment designed to remove or modify specific groups yields information in the form of missing and enlarged peaks. Treating the sample vapor with sodium metal will, for example, leave only hydrocarbons and ethers; hydrogenation (with platinum oxide as a catalyst) will saturate unsaturated compounds; treatment with hydrochloric acid will leave only neutral and acidic compounds.

In summary, GLC alone is never useful for the characterization of complete unknowns; used in conjunction with other analytical tech-

niques, it can be a useful aid. For qualitative analysis, GLC is most useful when linked to such analytical tools as infrared and mass spectrometers. In such cases, characteristic "fingerprints" of each GLC peak (on different columns) can be obtained, and frequently quite complex unknowns can be characterized.

QUANTITATIVE ANALYSIS

Gas chromatography is an extremely powerful tool for separation and quantitation of complex mixtures. It has revolutionized lipid chemistry in the last 15 years. It is, for example, possible with GLC to analyze mixtures of dozens of fatty acids in a relatively short time, an analysis which in the past would have taken months and even then probably would not have included trace components.

Certain rules must be rigidly observed during quantitative analysis by GLC, and time must be taken to standardize conditions. A little time spent in the beginning will pay dividends later in the form of smooth-running, routine, accurate analyses.

The first essential is investment in a set of highly purified standards. These standards should be analyzed to determine the linear dynamic range and response of the instrument. Correction factors should then be calculated for all types of columns to be used. Standards should be run periodically to check for changes in the instrument; they should always be run whenever a new column, new detector, or any new electronics part is fitted.

Before standardization of methods can begin, a method of peak measurement must be selected. The two methods available are peak height and peak area.

The measurement of peak height is the easier method provided that clear, sharp peaks are routinely obtained. In practice, this is seldom achieved for all compounds except when programmed temperature is employed. Peak height measurement under other conditions requires much standardization and is not recommended.

A number of techniques for measuring peak area are available:

(a) *Disc integrator.* This is an electromechanical device attached to the recorder. Over the bottom 10 percent of the recorder chart paper, the integrator pen travels with a speed proportional to the displacement of the recorder pen. This technique gives an accuracy slightly better than that in method (d) described below. It is especially useful when peaks are asymmetrical. It has the added advantage that a permanent record of quantitation is available on the original chromatogram. Peaks must stay on scale with this method, so integrator settings must be adjusted during a run or must be predetermined.

(b) *Electronic integrator.* These integrators usually integrate peaks and print out peak area and retention time. This is the quickest and most

accurate method of peak area measurement. Usually these devices correct for baseline drift, determine areas of peaks that are incompletely resolved, and operate independently of the recorder so that off-scale peaks are still measured. Not unexpectedly, these devices are quite costly.

(c) *Planimetry.* Peak area may be determined by tracing the peak periphery with a planimeter, a device that mechanically integrates the peak and records the area digitally on the planimeter dial. This technique can be quite accurate, depending upon the operator's skill.

(d) *"Height times width at one-half height" method.* Using this procedure one must first construct a baseline by drawing a line with a ruler across the bottom of a peak, making the best connection between the peak's leading and trailing edge. The peak's height, half-height, and width at one-half height are determined. The area is the product of the height and the width at one-half height. In other words, it is assumed that the peak is essentially a triangle. The usual formula for area of a triangle (½ bh) is not used, since the length of the base of a broad peak is often difficult to determine. This method works best when peaks are well resolved and fairly sharp and symmetrical. Increasing the speed of the recorder chart often improves peaks for this method.

(e) *Triangulation.* In this method a triangle is constructed by drawing tangents to the peak sides. The apex of the triangle will appear above the peak apex, but this allows for the area lost by drawing tangents to the sides. The base of the triangle is defined by the intercept of the tangents with the base line drawn across the bottom of the peak. The area of the peak is determined by the usual formula for area of a triangle, ½ bh. Trouble is encountered with this, as with the previous procedure, when peaks are asymmetrical or when peaks are incompletely resolved (the recorder pen does not return to the baseline between peaks). One way to overcome this is to measure the peak width at two points and calculate the area according to the formula:

$$\text{area} = \tfrac{1}{2} \times \text{height} \times (\text{width at } 0.2 \text{ height} + \text{width at } 0.8 \text{ height}).$$

This procedure is said to compensate for peak asymmetry and provide a more accurate area.

(f) *"Paper doll" technique.* In this method the peaks are cut out and weighed. This gives good reproducibility and is excellent for asymmetric peaks. The disadvantages are that the chromatogram is destroyed and the method is time consuming.

Standardization Procedures for Quantitative Analysis

Purity of standard compounds should first be assessed. As with all analyses, the standards should be run on two columns, one with a

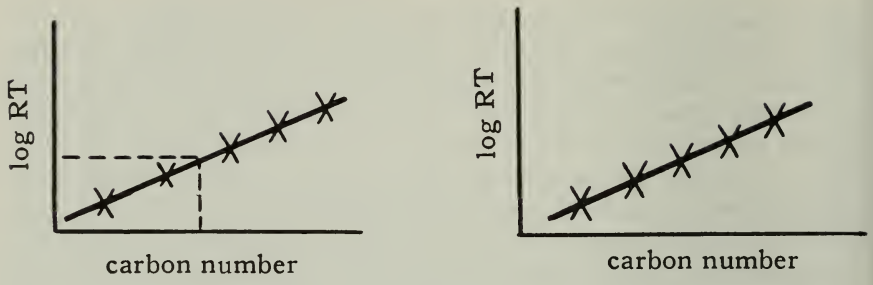
polar liquid phase (such as ethylene glycol succinate, EGS) and one with a nonpolar phase (SE-30 or an Apiezon). If the component gives a full-scale deflection on the recorder chart without the appearance of impurity peaks and if it has the correct RRT (on both columns), checked by referring to the literature, then it is about 99 percent pure. This, of course, assumes that impurities separate from the main component and is why at least two columns should be used to assess purity. If this condition is satisfied, then 10 times the amount can be chromatographed to establish 99.9 percent purity. Generally speaking, 99 percent purity is acceptable and the above procedure is adequate, provided that the standards have been purchased from one of the established suppliers. If the source of the standard is in some doubt, however, then establishing purity by GLC alone is insufficient. It must be remembered that the impurity may not be revealed by GLC. In such cases, it is advisable to use other approaches; for example, TLC in two different systems may be used to determine if only a single component is detected. If a lot of material is available, the compound should be examined by some other procedure such as characteristic infrared spectra, etc.

In the case of lipids it is seldom necessary to go to other analytical aids to assess purity when the source is one of the well-known lipid suppliers (such as Applied Science Laboratories, Mann Chemical Corporation, Sigma Chemical Corporation, Supelco, Inc.). These suppliers state the purity of their products and are on the whole reliable. Often chromatographic proof of purity is supplied with the more expensive standards. However, spot GLC checks should be run. If the compound is rare, detailed proof of purity should be obtained.

In place of the check on RRT's from the literature, another property, called the Carbon Number (CN) may be used. Within reasonable limits CN's are accurately reproducible for a particular liquid phase. The CN of a compound is found in the following way. Using fatty acid methyl esters as an example, assume that at least four standard esters have been run on a polar (EGS) and nonpolar (SE-30) column and that the retention times have been plotted on semilog graph paper versus the number of carbon atoms (here called the CN) in the ester. Figure 12 shows two typical graphs. If another ester, say methyl oleate, is now run on a column and its RT calculated, its CN can be read off the graph as shown by the dotted line. Lists of CN's are found in the literature and can be used both to help establish purity of standards and to identify fatty acids in a series being analyzed. It is advisable to establish the CN on both a polar and a nonpolar column.

Once purity of standards is established, the Detector Response (DR) and Linear Dynamic Range (LDR) should be determined.

Detector response must be determined so that the relationship between the amount of sample injected and the response of the detector

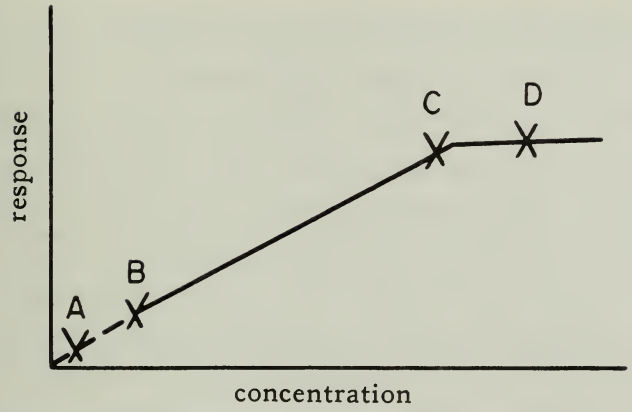


Determining carbon numbers (CN's) by chromatography on two columns. (Fig. 12)

is known. Unless good DR is obtained, analysis of small samples is not possible. DR varies with detector type, column, instrument make, and type of compound. To determine DR, a weighed mixture of standards A, B, C, and D is chromatographed. The weight injected must be known, and dilution of the mixture in solvent and measurement of injected volume (using a 10μl Hamilton Syringe) must be accurate. The injection should be repeated several times to check for reproducibility. The injection should be done steadily, but quickly, and the septum on the injection port must be leak free. After obtaining the chromatogram, the area of each peak is determined. The response of the detector to each compound per unit weight is then determined. The adjusted area of detector response is calculated from the relationship DR = area $\div$ weight. The response ratio is then calculated by selecting one compound as having a response of unity. Suppose compound B is our response standard. If one microgram of B gave peak area of 352 units (352 units per μg), then we divide the other DR's by 352 in order to obtain the ratio of response per μg to compound B. Alternatively, a factor can be obtained by dividing the other DR's by the DR of the compound selected as unity. Peak areas obtained on the chromatogram can then be corrected for variation in detector response by multiplying by the correction factor (or the response ratio) obtained.

The determination of response factors is time consuming, but in lipid chemistry some short cuts are usually possible. For example, many compounds of the same carbon length will have the same response factor. While the response factor for stearic (C18:0) acid may differ from that of oleic (C18:1) acid, other unsaturated acids of the same chain length may give the same response factor correction as oleic acid.

Linear Dynamic Range refers to the sample sizes that can appropriately be used on a given instrument, from the minimum detect-



Typical linear dynamic response curve for a given instrument. (Fig. 13)

able quantity to the point of overload. Ideally, if 1 μg of a sample gives a peak area of 10 units, 10 μg should give 100 units. Also, a plot of response versus concentration should pass through zero. In most cases these requirements are met, but the instrument's LDR must be checked. A linearity curve will show the concentrations at which the system becomes nonlinear. In addition, any interaction of the sample with the system (sample loss) will show up.

A typical LDR curve is shown in Figure 13. Point B is the minimum detectable level (response $\times$ 5 times the noise level); point C is the upper limit of linearity (frequently due to column overload); D is a typical point at which the concentration will not give valid results. An LDR curve which approximates an S-shape and has a narrow range (say from A to B) indicates sample adsorption by the column or other parts of the instrument. This type of LDR curve indicates a need for silylation of the column, use of a glass rather than a metal column, or both. If the curve crosses the Y-axis above zero, this can indicate either response when no sample is injected or sample measurement error on the part of the operator. If more than one effect is present at one time — say adsorption plus sample measurement error — a very distorted curve will be produced, and all possibilities must be considered. It is usually wise to consider sample measurement error first before changing column material.

Now that DR and LDR have been determined, one of the following standardization techniques can be chosen: internal normalization, external standardization, or internal standardization.

Internal normalization. In this procedure the quantity of a component in a mixture is expressed as a percentage of the total area of the chromatogram. Thus, if the sum of the areas of all the peaks is 100 and component A gives an area of 10, then the area percentage

for A is 10 percent. In calculating areas, the response factors for each component should be used to correct the areas. This technique is in common use and has a number of advantages — mainly, speed and independence from sample size. There are, however, several disadvantages: first, all peaks must be measured; second, absolute measurements rely on the assumption that the entire sample chromatographs; and third, for complete accuracy response factors for all peaks should be known. In practice these disadvantages are not very serious. Often in lipid chemistry one is attempting to completely analyze a series of monoglycerides, fatty acid methyl esters, etc., so one wishes to measure all peaks anyway. Such samples are usually prepurified (by TLC or some other method), and all components usually chromatograph. Indeed, it is common practice to convert peak area percentages into moles using the molecular weight — which assumes that everything chromatographs. Usually this is safe, but the investigator should ascertain that no adsorption is occurring.

Internal normalization is the most commonly used technique in GLC of fatty acid methyl esters and of dimethyl acetals (and other derivatives) of fatty aldehydes; it is also used in other lipid GLC work.

External standardization. This technique demands that a number of analyses of both the standard and sample be made and averages determined. The concentration of the standard should be near that of the sample or should be the same compound as the sample so that no response factor is required. Samples of known size must be injected; in practice this is hard to achieve, and the procedure is generally used only when a single peak is being analyzed. It is not recommended as a quantitative procedure for any of the lipid analyses discussed later in this section.

Internal standardization. This method is the best for obtaining accurate determinations by GLC and it is highly recommended whenever conditions permit its use. An example of internal standardization in cholesterol analysis by GLC is discussed in Chapter 6 (p. 87).

In this method, a known amount of a substance that is not present in the sample is added to an aliquot of the sample. The area of the peak of the added standard is determined and compared with the area of the sample component of interest. When the component is 1 to 100 percent of the sample, it is usual to use a weight of internal standard of about 10 percent of the weight of the component; for trace amounts, however, the standard should be in the same concentration range as the components.

It is not always possible to use internal standardization since suitable substances for standards are not always available. The standard chosen must fit certain specifications. It must elute from the column well separated from all sample components but near to them. It must have

functional groups similar to that of the sample component or be an appropriate hydrocarbon. It must be stable under the analytical conditions and not react with sample components. Finally, it must be sufficiently nonvolatile and stable to permit storage in solution for a significant period of time.

The first steps in internal standardization are preparing the standard solution and determining the response factor (RF) for the sample compound relative to the internal standard. A mixture of the component to be analyzed and the internal standard is prepared, and the RF is calculated as follows:

$$\text{RF} = \frac{\text{weight of internal standard} \times \text{area of component peak}}{\text{weight of component} \times \text{area of internal standard peak}}$$

Next, a sample mixture is prepared by adding a known amount of the internal standard to a known amount of the sample containing the component of interest. This mixture is then chromatographed under the same conditions as the standard and the percent of the component by weight is calculated by the equation:

$$\text{percent} = \frac{\text{component area} \times \text{internal standard weight} \times 100}{\text{RF} \times \text{internal standard area} \times \text{sample weight}}$$

Following the selection of an internal standard, it is necessary to determine the response characteristics of standard mixtures. A response versus concentration curve should be reproducible and preferably linear. The linearity plot should be linear over a wide concentration range and should finally plateau. In preparing this curve, the concentration of the internal standard is kept constant and only the concentration of the sample is varied.

CHEMICAL MODIFICATION OF COMPOUNDS FOR ANALYSIS BY GAS-LIQUID CHROMATOGRAPHY

Many lipids can now be analyzed by GLC in their unmodified state. Frequently, however, the preparation of a more volatile derivative, or one which has certain functional groups blocked, or both, leads to more efficient chromatography. Furthermore, chromatography of both the original and the chemically modified form will provide additional information about a sample and may serve as a check on quantitative procedures.

Analysis of Methyl Esters of Fatty Acids

Fatty acids are generally analyzed as their methyl esters,[1] although free fatty acids can be chromatographed. The method of methyl ester

[1] Other derivatives are employed; however, not enough is known about the quantitation of yields to rank these as competitors with methyl esters.

preparation varies with the source of material. Transesterification with acidic methanol is in common use. This approach gives the methyl ester of all the fatty acids in lipids from biological sources whether they are in amide (sphingolipid) or ester (glyceride and phosphoglyceride) linkages. Numerous procedures have been reported and a partial list of suitable reagents follows.

1. 1 to 10 percent sulfuric acid in methanol at 60 to 100°C for 1 to 16 hours.
2. 1 to 10 percent hydrochloric acid or 5 percent sulfuric acid } + 2,2-dimethoxypropane (as a water scavenger) in methanol at 50 to 70°C for 2 to 4 hours.
3. 3 to 14 percent boron trifluoride in methanol at 60 to 100°C for 1 minute to 16 hours.

Some workers carry out reactions by refluxing (under nitrogen), while others seal the reaction mixture in screw cap vials or glass ampules and heat in a water bath. Definitely avoid using hydrochloric acid-methanol, since this reacts to yield artifacts which mimic fatty acid esters on GLC (*1*).

The method we recommend uses 4 percent sulfuric acid in methanol in a sealed ampule at 90°C for 2 hours. Ten to 500mg of sample are placed in a 10ml glass ampule and an appropriate volume (0.5 to 10ml) of H_2SO_4-methanol is added. The ampule is cooled in dry ice-acetone and sealed in an oxygen flame. It is then heated for 2 hours in a water bath at 90°C (*Caution! Do not heat above this temperature or in an oven as the ampules will explode*). This procedure can be used directly on spots removed from a TLC plate if a noninterfering detection spray has been used. After heating, the ampule is again cooled in dry ice-acetone, opened, and 2 to 5ml water are added dropwise until two phases appear. The methyl esters are extracted with hexane or diethyl ether (3 × 3 volumes).

If there is doubt about the purity of the methyl ester preparations, they should be purified by TLC (in the presence of an antioxidant). For example, if total lipid from an animal source is transmethylated, the methyl esters must be purified by TLC since cholesterol derivatives that mimic fatty acid esters on GLC may appear; moreover, aldehydes, derived from plasmalogens, will form dimethylacetals under acid methanolysis conditions. These must be separated from the methyl esters by TLC or other means (see pages 78-79).

Generally, a good instrument will give a good resolution of the methyl esters of most natural fatty acid mixtures, and numerous column packings are available for this analysis. If good standards are available and semilog plots of RT versus carbon number are drawn (see pages 71-72), all major components can usually be identified. The ideal way to characterize complex mixtures is to make a preliminary separation by TLC first and then gas-chromatograph each fraction separately;

for example, mixtures may be separated into saturated and unsaturated fractions on silver nitrate impregnated plates as described earlier. If only small quantities of material are available, however, this approach may involve too many manipulations, and some losses may occur.

A previously uncharacterized mixture should be chromatographed on both a polar column, such as ethylene glycol succinate, and a nonpolar one, such as one of the Apiezon or SE-30 greases. On a polar column, it is usual for unsaturated fatty acid esters to elute after the saturated ones of the same carbon chain length, and branched chain esters are eluted just before the straight chain ester of the same carbon chain length. On nonpolar columns, unsaturated esters precede the saturated esters of the same carbon chain length.

Some coincident elutions often occur, and on many columns some unsaturated acids, especially those with many double bonds, may elute at the same time as a long-chain saturated acid. Uncharacterized mixtures must be checked for this possibility. If such mixtures were not initially separated by TLC, the sample should be treated in methanol with platinum oxide as a catalyst[2] to hydrogenate the fatty acid esters. The mixture should be chromatographed both before and after hydrogenation and on more than one kind of column. The appearance of new peaks, the change in size of old peaks, the disappearance of old peaks, and so forth will provide information about the presence of any unsaturated species.

The presence of hydroxy fatty acid methyl esters, eluted from many types of columns under certain conditions, can confuse identification of peaks. Mixtures containing hydroxylated fatty acids (for example, cerebroside and sulfatide fatty acids) should be separated into hydroxylated and nonhydroxylated ester fractions by one of the TLC or column chromatographic procedures described on pages 36 and 48. Derivatives of the hydroxylated esters can be prepared in which the free hydroxy groups are blocked. GLC of these derivatives — as trifluoroacetates, methyl ethers, or trimethylsilyl ethers — gives a much more rapid analysis and better peak symmetry. The third of these derivatives is highly recommended and easily prepared. Most suppliers of GLC accessories now offer a variety of "silylating kits," and while these are relatively expensive, they generally pay for themselves in the form of time saved. Usually all the investigator does is add a small volume of the silylating agent; after a short waiting period,

[2] Preferably, the hydrogen should be supplied via a manometer so that hydrogenation can be continued until there is no further uptake of hydrogen. The mixture should be stirred with a magnetic stirrer during the reaction. A simple, safe, and inexpensive hydrogenation apparatus has been designed by Applied Science Laboratories, Inc., State College, Pa. 16801. The apparatus is not offered for sale, but it is described in Applied Science's *Gas-Chromatography Newsletter* 10, No. 3 (1969). Further details can be obtained from the Biochemicals Department at Applied Science.

the mixture can be chromatographed directly without having to extract the derivative. If the hydroxy groups are sterically hindered, it may be necessary to heat the reaction mixture.

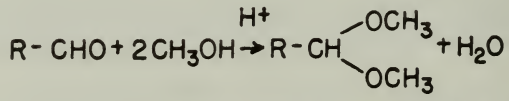
While many positional isomers of fatty acid esters separate under average conditions, geometric isomers do not. *Cis* and *trans* isomers can be separated efficiently with Golay (capillary) columns (see p. 97). Another approach is to convert the isomers to hydroxylated acids by oxidizing with osmium tetroxide (*2*). The hydroxylated compounds are then derivatized by one of the procedures mentioned above and chromatographed. After such treatment, the *erythro* and *threo* derivatives separate on the usual columns used for methyl ester GLC.

Quantitation of methyl ester analyses may be carried out using any of the peak measuring techniques previously described. Generally, it is wise to obtain response correction factors for all the saturated esters and for at least one unsaturated ester of each chain length.

For details of the GLC of some rare fatty acids, such as those containing acetylenic bonds, *Lipid Chromatographic Analysis,* vol. 1, should be consulted (see Suggested Further Readings).

Fatty Aldehydes

Usually the lipid chemist encounters fatty aldehydes in the form of their dimethyl acetals (DMA's), derived from the acid hydrolysis of plasmalogens. While aldehydes can be analyzed by GLC in their underivatized form, this is not a wise procedure. On standing, even for short periods, aldehydes readily undergo condensation and polymerization, reactions that are accelerated under alkaline conditions. It is preferable to obtain aldehydes in a stable form, such as the DMA, or to convert them to this or other stable derivatives as soon as possible. The DMA is the most satisfactory derivative since it is readily formed in quantitative yield and is very stable under neutral and alkaline conditions. Furthermore, if lipid mixtures are subjected to acid hydrolysis, fatty acids (whether in ester or amide linkages) are converted to methyl esters and plasmalogen aldehydes are converted to DMA's all in one step. Only a simple separation procedure must be employed, and both sets of derivatives can be subjected to GLC.



dimethyl acetal (DMA)

The methyl esters and DMA's may be separated either by TLC on silica gel using benzene as the developing solvent or by taking advantage of the fact that DMA's are stable in alkaline solution whereas

methyl esters are not. In the latter procedure, the methyl ester-DMA mixture is refluxed with 0.5N methanolic sodium hydroxide for two hours. The methyl ester fatty acids will be converted to sodium salts (soaps) and the DMA's will remain unchanged. On cooling, the solution is diluted with water (1 vol.) and the DMA's are extracted three times with hexane (1 vol.). The extract is washed with water-ethanol-3N sodium hydroxide 40:10:1 v/v/v (*3*). The combined hexane extracts are dried over sodium sulfate, the solvent is removed, and the acetals are reserved for GLC. The soaps in the water layer are converted to free fatty acids by acidifying the solution with 1N hydrochloric acid. The free fatty acids are then extracted with hexane, and, after drying, the solvent is removed. The fatty acids may then readily be reconverted to the methyl esters by any of the usual procedures (see p. 76).

Other derivatives of long-chain aldehydes that are useful for GLC analysis are the alcohol, the alcohol acetate, and the acid. Farquhar (*3*) used all these derivatives, in addition to the DMA's, in his detailed study of the fatty aldehydes of erythrocyte plasmalogens. He prepared the free aldehydes by dissolving the DMA's in 90 percent acetic acid 1:30 w/v and adding 1 drop of a saturated solution of mercuric chloride. The mixture was heated in a sealed ampule (under nitrogen) for 8 to 24 hours at 37°C. The aldehydes were recovered as follows: 1 volume of water was added; the solution was neutralized with 3N sodium hydroxide and extracted four times with petroleum ether; the extracts were washed once with water-ethanol-3N sodium hydroxide 40:10:1 v/v/v and dried over sodium sulfate. The solvent was then removed and the aldehydes were converted to derivatives as soon as possible.

The alcohols were prepared by reducing 3 to 30mg of the free aldehyde with 10ml of 3 percent lithium aluminum hydride *anhydrous* ether kept at −20°C for 2 hours. "Anhydrous" is stressed as the reaction of lithium aluminum hydride with water is *extremely violent.* The reaction was stopped by adding 5ml of water drop by drop to the reaction mixture which was still at −20°C. Five ml of ethanol were added, the supernatant ether was removed, and the lower layer was extracted 5 times with petroleum ether at room temperature. The combined extracts were washed with water-ethanol 4:1 v/v, dried, and the solvent removed.

One to 10mg of the fatty alcohols were converted to the acetates by dissolving in 9ml of acetic anhydride-pyridine 3:6 v/v in a glass-stoppered tube. The tube was kept at 37°C for 15 minutes and shaken occasionally. Five ml of water were added and the acetates were recovered by extracting 3 times with 5ml petroleum ether.

Conversion of the free aldehydes to the fatty acid is of limited usefulness since the usual oxidation procedures destroy the unsaturated

aldehyde. Farquhar (*3*) employed alkaline silver oxide as the oxidizing agent. Some information, however, can be derived from the fatty acids obtained from the aldehydes derived from the DMA mixture before and after hydrogenation. The DMA's can be hydrogenated in methanol using platinum oxide as a catalyst. It is usually necessary to add chloroform to the reaction mixture (up to 20 parts by volume) to keep the DMA's in solution. The fatty acid derivatives are generally prepared only when additional structural information is sought. For most purposes, the preparation of the DMA's or the alcohol acetates suffices for the GLC analysis of fatty aldehydes. If fatty acid derivatives are used, they can be converted to their methyl esters for GLC analysis.

A number of liquid phases are suited to the GLC analysis of DMA's and alcohol acetates. It is most important to remember that acidic columns must not be employed, as the DMA's will readily be hydrolyzed in acid conditions. Farquhar used ethylene glycol adipate (EGA) and Apiezon M for the analysis of the acetates, the DMA's, and the methyl esters. Other useful phases are ethylene glycol succinate (EGS), ethylene glycol succinate-silicone copolymer (EGSSX), Reoplex 400, and Apiezon L. Usually 10 to 15 percent EGA, EGS, EGSSX (polar) packings or 10 to 12.5 percent nonpolar packings is employed. Alkaline washed supports are recommended. Temperatures used with average columns (6 to 10 ft. × ⅛-inch) are about 150°C for polar phases and 190°C with nonpolar phases.

Aldehydes are not readily available in pure form, so standards for these analyses can present a problem. It is usual to use relative retention data and to refer all retention times to the normal C18 saturated hydrocarbon (octadecane) or, preferably, the corresponding 16-carbon derivative of hexadecanal (palmitaldehyde). The palmitaldehyde is available in a relatively pure state as its bisulfite. The bisulfite is converted to the DMA by heating with 4 percent sulfuric acid in methanol, or the free aldehyde is obtained by aqueous acid hydrolysis. The DMA preparation can be freed from aldehyde by shaking the extract with a saturated solution of sodium metabisulfite. The DMA extract should also be washed with an alkaline wash solution (water-ethanol-3N sodium hydroxide 40:10:1 v/v/v) according to Farquhar's procedure to prevent acid-catalyzed breakdown. Retention times of components are stated as relative to hexadecanal, hexadecanal acetate, hexadecanal DMA, or methyl hexadecanoate, according to the derivative chromatographed, since long-chain aldehyde GLC data is generally so reported in the literature; this permits ready comparisons with the work of others.

On polar columns, the order of elution of fatty aldehydes and their derivatives of the same carbon chain length (unbranched) is: free aldehyde, DMA, methyl ester of fatty acid, alcohol acetate, free alcohol. On a nonpolar column the order is: free alcohol, methyl ester, DMA, and alcohol acetate. Free aldehyde is omitted from the last series be-

cause according to Farquhar, it was not eluted from an alkaline treated nonpolar column. More recently, Gray (*4*) has reported aldehyde chromatography on untreated Apiezon L, but under these conditions breakdown of DMA's occurred.

Peak area calculations may be made by any of the methods previously described.

Glycerides

The beginner in gas chromatography can, after a short practice period, expect to achieve reasonably successful analysis of uncomplicated fatty acid and fatty aldehyde mixtures. This is not so, however, in the case of glyceride analysis since the situation is complicated by the high molecular weights of the compounds and by the complexity of the molecular species found in naturally occurring glycerides. Glyceride analysis by GLC is still in its infancy. The first practical demonstration of glyceride analysis (*5*) by GLC was shown one decade after the introduction of GLC analysis. Although glyceride GLC analysis is beyond the scope of this book, we can appropriately include a brief account of the difficulties and approaches to success in this area.

So far we have considered GLC analyses that can be readily accomplished using isothermal conditions. In general, the efficiency achieved under isothermal operation is not conducive to satisfactory analysis of glycerides. The efficiency of a GLC column isothermally operated is given by the number of "theoretical plates" (a term allied to distillation theory), for which the expression is: $N = 16(t_r/w)^2$. N is the number of theoretical plates, t_r is the residence time of the component in the column, and w is the width of the peak. An efficient column gives peaks of narrow width. If band diffusion is large and broad peaks result, programmed temperature is called for. A column operated isothermally under linear temperature programming conditions may often give 5 or 6 times the apparent number of theoretical plates. This improvement is very important to the chromatographer of glycerides. The best conditions determined so far for glycerides are low liquid phase concentrations; narrow, short columns; and temperature programming from 200 to 350°C. At temperatures above 350°C thermal cracking may result.

Triglycerides can be chromatographed directly, but in the interests of increased stability and volatility, mono- and diglycerides should be converted to suitable derivatives such as acyl esters or silyl ethers. Isopropylidene and benzylidene derivatives have also been used.

The usual columns employed for glyceride analysis (triglycerides or derivatized mono- and diglycerides) are the silicone polymers (Se-30), polysiloxane polymers (JXR, Applied Science Laboratories, Inc.), and fluoroalkyl silicone gums (Dow-Corning). The percentage of liquid phase employed does not usually exceed 3 percent and is frequently below 1 percent.

Because column "bleed" at high temperatures is considerable, instruments with "dual column operation" are preferable. Dual column operation usually compensates for baseline drift due to bleed by splitting the effluent stream and using dual compensating flame ionization detectors.

Unless some preliminary separations are performed, chromatography of glycerides yields peaks of mixed molecular species, generally classified according to the total number of carbon atoms they contain. If detailed molecular species analyses are sought, then mixtures must be subjected to preliminary separations by column and thin-layer chromatography. Mixtures must be separated not only into mono-, di-, and triglyceride fractions, but also into fractions based on the degree of unsaturation. This may be achieved by argentation chromatography.

Acetate derivatives. The determination of phosphoglyceride structure has been discussed previously (Chapter 4, p. 55). This analytical procedure included the preparation and analysis of the diglyceride acetates derived from the phospholipids. These acetate derivatives are readily prepared at room temperature by dissolving about 5mg glyceride in 1ml dry pyridine (distilled over barium oxide) in a tight screw cap vial and adding 0.5ml acetic anhydride. The mixture is allowed to stand overnight, after which excess reagent is removed by evaporation under vacuum. Alternatively, diglyceride analysis may use silyl ether derivatives prepared with one of the available silylation reagents. Whichever derivative is chosen, the analysis can be carried out (isothermally or by programmed temperature operation) on column packings previously mentioned (for example, 1 to 3 percent SE-30, 3 ft. $\times$ $\frac{1}{8}$-inch column) at temperatures between 180 and 300°C.

The preceding comments merely cover some of the major points regarding GLC of glycerides. The reader interested in pursuing this field is referred to the Suggested Further Readings.

Other Lipids and Components Derived from Lipids

Other nonpolar lipids, such as the glyceryl ethers, can also be subjected to GLC analysis usually as trifluoracetate or silyl ether derivatives.

Glycerol, amino alcohols such as sphingosine and related compounds, and carbohydrates derived from lipids can also be analyzed by GLC generally in derivatized form. References to the GLC analysis of nonlipid moieties of lipid molecules are given in Suggested Further Readings.

PYROLYSIS-GLC

The GLC analysis of the products of pyrolysis has been used advantageously in the study of hydrocarbons. Recently, several investigators have started to explore the use of pyrolysis-GLC as an approach to the determination of phospholipid structure. Kuksis, Marai, and

Gornall (*6*) noted that serum lecithins undergo pyrolysis in a flash evaporator (at 280 to 300°C) attached to a GLC apparatus. They tentatively identified the chromatographed peaks as the propenediol diesters, which under most conditions have the same retention times as the corresponding diglycerides. Perkins and Johnston (*7*) subjected a number of phosphoglycerides to pyrolysis-GLC and made a mass spectral study of the products. All the phosphoglycerides were found to cleave at the phosphate ester bond, and the GLC peaks obtained had retention times the same as those of the corresponding diglycerides. The mass spectral data confirmed the elimination of the phosphate ester group and showed that the products obtained were the dehydrated diglycerides, that is, the diacylesters of propenediol. A different approach has been used by Horning, Casparrini, and Horning (*8*), who subjected phospholipids to silylation with bis-trimethylsilylacetamide (BSA) and trimethylchlorosilane (TMCS) and injected the derivatives into the gas chromatograph. They identified the products of the phosphoglycerides as the corresponding dehydrated trimethylsilyl derivatives of the diglycerides. These investigators also found that the phosphate ester group was eliminated from silylated sphingomyelin on thermal cracking, and that trimethylsilyl derivatives of ceramides amenable to GLC and GLC-mass spectrometry studies could be obtained.

Further studies are needed to explore the possibility of adapting these findings to the analysis of polar lipids by GLC.

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VI. Procedures for the Determination of Specific Elements, Functional Groups, and Lipid Classes

In this chapter the determination of specific elements, functional groups, and individual lipids are described in detail. These procedures allow the investigator to determine the amount of specific lipids (cholesterol, gangliosides) and specific lipid classes (total phospholipid, glycolipid, etc.) in lipid mixtures. They can also be applied to the quantitative analysis of lipids separated by column, thin-layer, and paper chromatographic procedures.

In keeping with all former accounts in this text, this chapter is not a compilation of all available methods for these determinations. The methods presented are selected on the basis that the author or a colleague has had considerable experience with them and has found them to be satisfactory.

DETERMINATION OF ORGANIC PHOSPHORUS

The method described is a modification of Bartlett's (*1*) procedure.

Reagents: concentrated sulfuric acid
70 percent perchloric acid
ammonium molybdate, 0.26 percent aqueous solution
disodium hydrogen phosphate (for standard curve)
Fiske-Subbarow Reagent

Equipment: pyrex test tubes
water bath and sand bath
spectrophotometer reading at 800 to 820mμ

Fiske-Subbarow Reagent: To 80ml of 15 percent sodium bisulfite add 0.2g purified 1-amino-2-naphthol-4-sulfonic acid and 0.4g anhydrous sodium sulfite, while stirring. Filter and store in brown bottle. Make fresh solution each week.

Procedure: To a test tube containing 0.5 to 20.0μg of lipid phosphorus add 0.5ml concentrated sulfuric acid. Digest the lipid by placing the tube in a sand bath at 250°C for 3 hours. Complete the digestion by adding 3 drops (approx. 0.15ml) 70 percent perchloric acid and heat at 250°C for a further 30 minutes. Mix the contents of the tube occasionally. After digestion is complete (solutions should be clear), cool the tubes and add 9.1ml of 0.26 percent ammonium molybdate and 0.4ml Fiske-Subbarow reagent. After the solution has been mixed

thoroughly (preferably with a Vortex mixer), heat the tubes in a bath of boiling water for 10 minutes, then cool. Read the blue color developed at 800 or 820mμ; the latter is preferred if available. Zero the instrument with a prepared reagent blank.

Prepare a standard curve 0.2 to 30μg of phosphorus, using potassium dihydrogen phosphate.

Application of method to spots on TLC plates. This procedure can also be applied directly to adsorbent plus lipid removed from a TLC plate. The digestion will go to completion in the presence of adsorbent (preferably Silica Gel H) and remaining traces of solvents such as n-butanol. The solution will clear when digestion with perchloric acid is complete.

Before deciding on a loading for a TLC plate, the total lipid phosphorus should be determined and a trial TLC plate should be run to determine how many phospholipids are present. The relative concentrations of the phospholipids may be judged by charring. A suitable loading can then be selected. A load which gives about 0.5 to 15.0μg lipid phosphorus per spot is ideal; however, the method will detect amounts from as little as 0.1 to 0.2μg up to 25.0μg.

After developing the plate, spot an amount of total lipid containing 5 to 10μg phosphorus on a part of the plate untouched by solvent, and remove the spot to make a total lipid phosphorus check. Also remove a clear area of equal size to the blank. Spots are best visualized by exposing the plate to iodine vapor in a closed jar, after the solvents have evaporated. Spots of interest can be outlined with a needle, removed either by aspiration or scraping, and placed in test tubes. Remove clear areas of equal size and place in test tubes to use as sample blanks. The procedure is carried out as described above, except that after color development the tubes must be centrifuged at about 600 × g and the solutions decanted into clean tubes to free them of the silica gel. The color is read in the usual way, subtracting adsorbent blank optical density from the sample before reading off the standard curve. Blank readings above optical density of 0.03 to 0.04 are undesirable and indicate contamination of either the adsorbent or reagents with a phosphorus containing compound. To avoid contamination, wash all glassware in chromic acid and rinse thoroughly in deionized water. It may prove necessary to wash the Silica Gel H as described on pp. 42-43.

DETERMINATION OF CHOLESTEROL

By Spectrophotometry

The procedure is that of Zak, Luz, and Fisher (*2*).

Reagents: ferric chloride stock solution — 700g ferric chloride ($FeCl_3 \cdot 6H_2O$) in glacial acetic acid in a 100ml volumetric flask; dilute to mark and mix well.

ferric chloride working solution — stock solution diluted 1:10 v/v with glacial acetic acid.

cholesterol stock standard — 100mg pure cholesterol in glacial acetic acid in 100ml volumetric flask; dilute to mark.

digitonin solution — 1g digitonin in 50ml ethanol, diluted with distilled water to 100ml in volumetric flask.

acetone-alcohol 1:1 v/v
acetone
concentrated sulfuric acid

Equipment: spectrophotometer and 1cm cuvets
conical centrifuge tubes, 15ml
test tubes, 15ml

Procedure: Prepare a standard curve by diluting 1ml of the ferric chloride stock solution and 1ml of the cholesterol stock standard with glacial acetic acid to 10ml in a volumetric flask. Pipet 1.0, 2.0, and 3.0ml of this standard into test tubes, diluting the 1.0 and 2.0ml fractions to 3.0ml with the ferric chloride working reagent. Prepare one blank tube of 3ml ferric chloride working reagent. Carefully layer 2ml of concentrated sulfuric acid over each solution, then mix well. When the solutions have cooled to room temperature, measure absorbancies at 560mμ against the blank.

To determine serum cholesterol levels, pipet 0.2ml of serum into a 10ml volumetric flask containing approximately 5ml of acetone-alcohol solution. Dilute to mark with acetone-alcohol and shake vigorously to extract the cholesterol. Filter mixture through Whatman No. 41 filter paper, keeping a watch glass over the funnel. Pipet 5ml of filtrate into conical centrifuge tube for determination of free cholesterol and 2.5ml of filtrate into a test tube for total cholesterol determination. Evaporate contents of test tube to dryness and contents of centrifuge tube to 0.5 to 1.0ml.

To test tube add 3ml of ferric chloride working solution to dissolve residue. Layer in 2ml of concentrated sulfuric acid and mix solution well. Wait 15 minutes for color development, then measure the absorbance against a blank at 560mμ.

To the centrifuge tube add 0.5ml of digitonin solution, wait 15 minutes, then centrifuge at 600 to 700 $\times$ g for 10 minutes. Decant the supernatant. Add 4ml of acetone to disperse the precipitate, tapping the tube until the precipitate is homogenous, then centrifuge again for 10 minutes. Decant the wash solution. Invert tube on absorbent paper to drain. Add ferric chloride (3ml) and sulfuric acid (2ml) as described above, wait 15 minutes, and read absorbance at 560mμ.

The method has been shown to give stoichiometric final color reaction with amounts of cholesterol up to 1000mg per 100ml. The range of analysis can be extended by appropriate dilution of the original

sample. While the method has been described for analysis of serum cholesterol levels, it can, of course, be adapted to the analysis of the cholesterol content of any sample of total lipid. All that is required is dilution of the original sample to give a cholesterol content within the range 0 to 1000mg per 100ml.

By Gas-Liquid Chromatography

The method described utilizes cholestane as an internal GLC standard (*3*).

Reagents: cholesterol
cholestane
95 percent ethanol
diethyl ether
hexane
chloroform
potassium hydroxide

Equipment: 18mm × 150mm test tubes
serum caps to fit test tubes
centrifuge tubes, 15ml, screw cap
20ml vials
5 and 10ml pipets
graduated pipets
gas chromatograph equipped with glass column, glass inlet system, and flame ionization detector

Procedure: Prepare the following standard solutions:

1. Ratio Solution. Weigh 100mg cholesterol and 100mg cholestane. Dilute to 100ml with chloroform. Mix and store in refrigerator. These compounds are stable for several weeks.

2. Potassium hydroxide. Weigh out 3.3g potassium hydroxide and dilute to 10ml with distilled water. Prepare fresh daily.

3. Internal standard. Weigh 100mg cholestane and dilute with chloroform to 100ml. Subdivide solution into 10ml portions. Store in refrigerator in tightly stoppered containers.

Operating conditions for gas chromatograph:

Column. 18-inch glass packed with 3.8 percent SE-30 on Diatoport S. (Note: This column is recommended in the original procedure, but other columns are also suitable, such as 1 to 2 foot columns packed with 1 to 3 percent SE-30 on various supports.)

Column temperature. 200 to 230°C, depending on column packing, etc. Other settings will vary from instrument to instrument and must be determined individually.

Detector. A flame ionization detector is recommended because its response is usually linear over a wide range. Use of

another kind of detector will probably necessitate preparing a correction curve for the specific detector.

Determination of total serum cholesterol

1. Measure 0.2ml serum into test tube.
2. Add 4.7ml 95 percent ethanol.
3. Add 0.3ml 33 percent aqueous potassium hydroxide solution.
4. Stopper tube and mix well.
5. Place in water bath at 55°C for 15 minutes.
6. Remove tube and cool.
7. Add 5ml distilled water.
8. Add 10ml hexane and allow to stand till two phases appear.
9. Remove a 5ml aliquot of the upper (hexane) phase and transfer to a 3-dram screw-cap vial.
10. Use a stream of nitrogen and a water bath to evaporate the hexane aliquot to dryness.
11. Add 0.2ml of the internal standard solution to the dry residue.
12. Cap vial and mix well; inject about 3μl of mixture into gas chromatograph (*exact size* of sample is not important when internal standardization is used — see p. 74).

Analysis on GLC will take 7 to 15 minutes, depending on the column.

Determination of free cholesterol

1. Measure 0.2ml serum into 15ml screw cap centrifuge tube.
2. Add 9ml 95 percent ethanol.
3. Add 3ml diethyl ether.
4. Shake and allow to stand for 5 minutes.
5. Centrifuge (600 to 700 $\times$ g).
6. Pour supernatant into 20ml vial.
7. Evaporate residue to dryness as previously described.
8. Add 0.2ml of internal standard solution.
9. Inject about 3μl into gas chromatograph; GLC analysis time will be the same as for total cholesterol.

Interpretation of chromatograms. First obtain a chromatogram by injecting 3μl of the cholestane-cholesterol standard solution and calculate the ratio either by using peak height or peak area, thus:

$$\frac{\text{cholestane peak height (area)}}{\text{cholesterol peak height (area)}} = \text{R}$$

To calculate total cholesterol in the sample, measure peak heights (or areas), multiply cholesterol peak height by the ratio, and divide by the cholestane peak height. Since in the original procedure 5ml of hexane was withdrawn from the mixture, the value must be multiplied by 2. Thus, the calculation for total cholesterol in a sample becomes:

$$\frac{\text{cholesterol peak height}}{\text{cholestane peak height}} \times R \times 2 = \text{total cholesterol (mg/100ml)}$$

To determine free cholesterol, follow the same calculation procedure as for total cholesterol. Since the whole sample is used in the analysis for free cholesterol, the calculation will be:

$$\frac{\text{cholesterol peak height}}{\text{cholestane peak height}} \times R = \text{free cholesterol (mg/100ml)}$$

The ratio R should be determined from standards daily, as slight changes in column conditions may affect it. It must also be remembered that if changes in sensitivity factors have been made during the run, these must be taken into account in the calculations.

THE DETERMINATION OF GLYCOLIPID SUGARS

By Using Anthrone

The method described is that of Radin *et al.* (*4, 5*).

Reagents: anthrone stock solution — 2 percent anthrone (recrystallized) in sulfuric acid; age 4 hours at room temperature (stable for 2 weeks in refrigerator).

anthrone working solution — stock solution diluted 14 times with sulfuric acid-water 9:5 v/v; make up just before use.

galactose standards — dry galactose at 100°C over phosphorus pentoxide and dissolve in water to give a concentration of 20mg per ml; store this solution in polyethylene bottle in refrigerator; prepare standards by evaporating appropriate volumes to dryness in colorimeter tubes.

hydrolytic solvent — ethanol-concentrated hydrochloric acid 74:63 v/v.

Equipment: colorimeter
screw-cap tubes, 10 to 15ml
constant temperature bath set at 58°C

Procedure:

1. Evaporate a sample solution containing 0.2 to 1.7mg cerebroside (or other galactolipid) to dryness in a screw-cap tube.
2. Add 3ml hydrolytic solvent and place in water bath at 58°C for 3 hours.
3. Add 5ml toluene (as an anti-splashing agent) and evaporate to dryness under vacuum.
4. Add 10ml water and 2ml chloroform, cap the tube, and centrifuge at 600 to 700 × g.

5. Transfer duplicate 2ml aliquots to colorimeter tubes, add 1ml toluene, and evaporate to dryness.

6. Add 5ml of the anthrone working solution and mix well.

7. Develop the color (green) by heating for 6 minutes at 100°C or 16 minutes at 90°C.

8. Read the color at 625mμ against a blank anthrone solution.

9. Prepare a standard curve from evaporated aliquots of standard galactose solution (as described above) treated as in steps 5 through 8.

If the complete fatty acid composition of the glycolipids is known, the average molecular weight can be determined and the galatose content can be used to calculate moles of glycolipid. If this information is not available, conversion of sugar content to quantity of glycolipid can be calculated on the basis of expected average sugar content for the particular glycolipid.

By Gas-Liquid Chromatography

Carbohydrates liberated from glycolipids can readily be analyzed by GLC. Preparation of the trimethylsilyl derivatives of sugars is quite easily and rapidly achieved with the commonly used silylating agents such as hexamethyldisilazane and trimethylchlorosilane in dry pyridine. Quantitative yields of the polytrimethylsilyl derivatives can usually be obtained within a few minutes at room temperature. The derivatives can be separated on short columns packed with nonpolar phases such as SE-30 (2 to 3 percent) at temperatures about 140°C to 160°C. Mixtures of sugars can be quantitatively determined by using the internal normalization procedure, and a single sugar can be analyzed by using an appropriate internal standard. However, while the preparation of derivatives and their GLC analysis are relatively simple matters, the quantitative liberation of the sugars from the glycolipids presents difficulties. Under some hydrolytic conditions (such as aqueous acid), some sugars may degrade. These difficulties have been discussed in detail recently by Sweeley and Vance (*6*). The best method available at present appears to be anhydrous methanolysis: the products liberated from most glycolipids under these conditions are equilibrated anomeric mixtures of relatively stable methyl glycosides that can be readily converted to derivatives for GLC analysis.

DETERMINATION OF N-ACETYLNEURAMINIC ACID (IN GANGLIOSIDES)

By Using Resorcinol

The method described is from Svennerholm (*7, 8*).

Reagents: hydrochloric acid, density 1.19 (at least 36.4 percent), Fe^{+++} less than 0.0001 percent.

0.1M solution of copper sulfate.

resorcinol stock solution — 2g resorcinol in 100ml deionized water (stable for months in refrigerator).

resorcinol working solution — 10ml stock solution added to 80ml concentrated hydrochloric acid that contains 0.25ml of the 0.1M copper sulfate solution (stable one week in refrigerator).

blank — same as working solution, without resorcinol.

Equipment: spectrophotometer
centrifuge
water bath
test tubes, 10 to 15ml

Procedure: Three 2ml samples containing 5 to 30μg of N-acetylneuraminic acid are pipetted into test tubes. Two of the samples are the unknowns in duplicate, and the third is the blank. To the two unknowns add 2ml of the working resorcinol reagent, and to the third tube add 2ml of blank sample reagent. Prepare a standard curve using N-acetylneuraminic acid in the range 0 to 30μg. Prepare as for samples using the 0μg tube as the blank. Heat the tubes for 15 minutes in a bath of boiling water. Cool and place in ice bath. Extract the color with 4ml of a solution of n-butylacetate-n-butyl alcohol, 85:15 v/v. Shake well and allow to settle in the cold, or transfer to centrifuge tube and centrifuge at 300 to 400 $\times$ g in a cold room or a refrigerated centrifuge. Complete this part of the procedure within 1 hour of the heating step. Read absorbance at 580mμ in 1cm cells. Subtract absorbance of blank sample from test samples and read μg of N-acetylneuraminic acid from curve. If greater sensitivity is desired (0.05 to 0.50μM of N-acetylneuraminic acid), the color may be read in 50mm microcells.

If this procedure is applied to gangliosides that have been separated into mono-, di-, and trisialogangliosides (compounds containing 1, 2, and 3 moles of N-acetylneuraminic acid per mole of ganglioside), then the N-acetylneuraminic acid content may readily be used to calculate the weight of individual gangliosides. A typical preparation of total gangliosides extracted from mammalian brain contains 24 to 27 percent N-acetylneuraminic acid.

This procedure can be applied to glycolipids containing N-glycolylneuraminic acid in place of N-acethylneuraminic acid; however, either a standard curve using the N-glycolyl compound must be used or the absorbancies must be corrected for the 30 percent greater molar absorbancy index of the glycolyl derivative. Values read from the N-acetylneuraminic acid standard curve would have to be multiplied by 0.77.

By Gas-Liquid Chromatography

The hydroxyl groups on N-acetylneuraminic acid can readily be silylated to yield a compound amenable to GLC analysis. Thus, the

methyl ester of N-acetylneuraminic acid, obtained on methanolysis of gangliosides, can be converted to the trimethylsilyl derivative with any of the available silylating reagents. This product can be subjected to GLC on a column such as 2.5 to 3 percent SE-30. For quantitative determinations it is necessary to use the internal standard technique (p. 87), as described for the GLC determination of cholesterol. The choice of a suitable internal standard for N-acetylneuraminic acid determinations has not been extensively studied. It appears, however, that a sugar such as mannitol or a suitable amino sugar should prove satisfactory.

THE DETERMINATION OF PLASMALOGENS

By Colorimetry

The method described is that of Gray and Macfarlane (*9*). It is a two-stage reaction procedure in which the aldehyde is first split off the plasmalogen and then condensed with fuchsin reagent to give a color.

Reagents: fuchsin reagent — dissolve 1g rosaniline hydrochloride in 700ml boiling water; filter, cool; add 5.0g sodium metabisulfite and 100ml of 1N HCl and make up to 1 liter with water; decolorize for 48 hours and store at 2°C in a dark, glass-stoppered, narrow-necked bottle.

sulfite water — 0.5 percent sodium metabisulfite in 0.1N HCl.

octan-2-ol (capryl alcohol), low ketone grade.

dimethyl acetal of palmitaldehyde, prepared from commercially available palmitaldehyde bisulfite (see p. 80).

acetic acid-water 90:10 v/v.

Equipment: spectrophotometer

15ml centrifuge tubes, glass-stoppered

Procedure:

1. To determine total aldehyde in lipid samples, prepare a stock standard solution by dissolving 48mg palmitaldehyde dimethyl acetal in 20ml chloroform; prepare a working standard by diluting to 100μg palmitaldehyde per ml. Prepare a standard curve of palmitaldehyde in the range 5 to 50μg. Place suitable amounts of the working standard in centrifuge tubes and remove solvent. Add 0.5ml 90 percent acetic acid and store at 50°C for 45 minutes, prepare one blank). Add 2.0ml fuchsin reagent, and after 20 minutes at room temperature add 2.0ml sulfite water and 5.0ml capryl alcohol. Shake vigorously and centrifuge for 4 minutes. Read the optical density of the colored layer against the blank at 546mμ. Now place an amount of lipid sample containing 5 to 45μg of aldehyde in centrifuge tubes and procede as with the working standard.

2. Determine the phosphorus content of the samples (see p. 84).

The ratio of moles of phosphorus to moles of aldehyde in a plasmalogen is unity. The ratio of moles of aldehyde in the sample (from step 1) to the moles of phosphorus in the sample (from step 2), multiplied by 100, gives the "Plasmalogen Value," or the percent of phosphorus that is present in the sample as plasmalogen.

By Two-Dimensional Thin-Layer Chromatography *(10)*

This method is based on the specific hydrolysis of plasmalogens to the 2-acyl lysophosphoglyceride in the presence of a mercuric chloride spray reagent.

Reagents: chloroform-methanol-water 60:35:8 v/v/v
chloroform-methanol-water-acetic acid 65:43:3:1 v/v
petroleum ether (bp. 40 to 60°C)-diethyl ether-acetic acid 80:20:1 v/v/v
aqueous ammonia (sp. gr. 0.880)
5mM mercuric chloride in deionized water
18N sulfuric acid

Equipment: 20cm × 20cm TLC plates
TLC spreader and board
Silica Gel H (E. Merck A.-G., Darmstadt, Germany)
10ml stoppered tubes
oven at 180°C, situated under extractor fan

Procedure: Prepare TLC plates (about 500mμ thick) using a Silica Gel H slurry prepared in ion-free water. Before activating plates at 110°C, wash the adsorbent by allowing chloroform-methanol-water (60:35:8) to ascend to the top of the plate. This removes material which interferes with charring by sulfuric acid. Silica gel washed in bulk may be used, but remember that separations will vary and the variations must be ascertained in advance for each silica gel used.

Duplicate lipid samples (containing 0.2 to 0.5μg atom of phosphorus) are plated as 1cm bands, one 2cm in from the left and the other 3cm in from the right-hand edge. Develop the plate in the freshly prepared chloroform-methanol-water-acetic acid 65:43:3:1 v/v to 8cm from the top of the plate. After removing the solvent, redevelop the plate to the top using petroleum ether (b.p. 40 to 60°C)-diethyl ether-acetic acid 80:20:1 v/v/v. This solvent mixture causes the lipids, other than phospholipids and glycolipids, to migrate to a position above the first solvent front. The residual acetic acid left on the plate after development is neutralized by supporting the chromatogram above an aqueous ammonia solution (sp. gr. 0.880) in a sealed dish for 5 minutes. The excess ammonia is then drawn off under vacuum (0.5mm mercury or less) for 30 minutes. The left-hand lipid track is then sprayed with 5mM mercuric chloride while the remainder of the chromatogram is screened. The plate is turned through an angle of 90° and the second solvent front is marked at 5cm from the top of the plate. The

plate is then reactivated by evacuating at 0.5mm mercury or less over dark blue self-indicating silica gel for one hour. The mercuric chloride-treated lipids are developed in the second dimension with chloroform-methanol-water 60:35:8 v/v/v. The chromatogram is then dried, sprayed with 18N sulfuric acid, and charred at 180°C for 1 hour in an oven situated beneath an extractor fan to ensure removal of volatilized sulfuric acid and mercuric chloride.

The charred areas are removed and placed in 10ml stoppered tubes. Appropriate blank areas (corresponding to large, medium, and small lipid spots) are also taken and are added to tubes containing evaporated aliquots (0.1ml) of a standard phosphate solution equivalent to 0.05μM of phosphorus. Total phosphorus is then determined on all samples and on blank plus standard samples. The phosphorus determination procedure used by the originator of this method was that of Sloane-Stanley and Eldin (*11*), but other procedures, such as the one previously described (pp. 84-85), can be applied.

The total lipid phosphorus content of the original diacyl and monovinylether-monoacyl phospholipid mixture plus the 2-acyl lysophospholipid left after hydrolysis with mercuric chloride is determined. These values for percent phosphorus can be converted to amounts of phospholipid by multiplying by an appropriate factor. The factors can be determined from the actual fatty acid composition of each phospholipid as determined by GLC. However, for many purposes the factors given by Williams *et al.* (*10*) for human serum phospholipids may be sufficiently accurate:

phosphatidyl choline	26.2
phosphatidyl ethanolamine	25.0
phosphatidyl serine	25.7
lysophosphatidyl choline	17.5
lysophosphatidyl ethanolamine	16.0

If the fatty acid composition of the lipids differs markedly from that of serum phospholipids and would mean fairly large differences in molecular weight, then factors for the lipids being analyzed must be determined.

When the percentages of the intact and the 2-acyl lysophospholipids are known, the percent plasmalogen can be determined by the difference between the two.

DETERMINATION OF THE AMOUNT OF *TRANS* DOUBLE BOND

By Infrared Spectrophotometry

Compounds with isolated *trans* double bonds exhibit an absorption band in the infrared due to a C-H deformation about the *trans* double bond. This absorption band has its maximum at about 10.3μ and can be used to determine quantitatively the isolated *trans* double bond

content of compounds. The procedure outlined below is the one described in *The American Oil Chemists' Society Handbook of Official and Tentative Methods,* vol. 1. The method is not applicable to lipids that contain large quantities of conjugated unsaturation, to compounds that contain functional groups that modify the intensity of the C-H deformation about the *trans* bond, nor to compounds which give rise to absorption bands near 10.3μ. For example, long-chain fatty acids with less than 15 percent isolated *trans* isomers must be converted to their methyl esters for analysis, because efficient correction for an absorption band of the carboxyl group at 10.6μ is not possible at that concentration of *trans*.

Equipment: any infrared spectrophotometer covering the region 9 to 11μ (900 to 1150cm^{-1}) with a wavelength scale readable to 0.01μ and fitted with a cell compartment for holding 0.2 to 2.0cm cells.

fixed thickness absorption cells with NaCl or KBr windows from 0.2 to 2.0cm.

Reagents: carbon disulfide (dry, ACS)

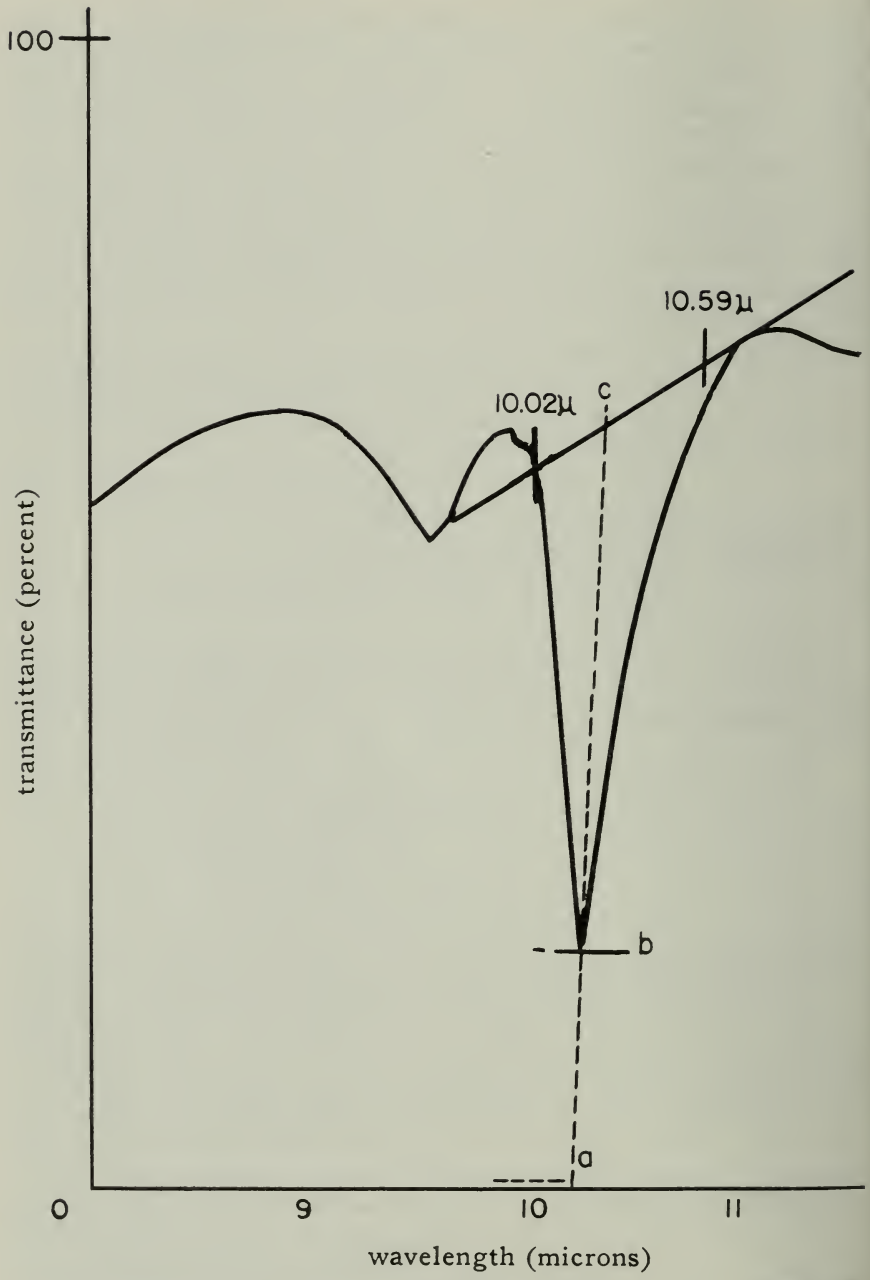
standards of elaidic acid, methyl elaidate, and trielaidin

Procedure: Weigh 200mg of standard; place standard and sample into a 10ml volumetric flask. Dilute to volume with CS_2 and mix. Transmittance at the *trans* absorption maximum should be 20 to 70 percent; if not, use a different sample weight or cell thickness.

Using matched absorption cells, fill one cell with carbon disulfide and the other with sample or standard solution. Place the cells in the reference and sample beam holders in the spectrophotometer. Measure the transmittance or absorbance over the range 9 to 11μ. Different instruments will require different programs in order to obtain a satisfactory curve. Once a satisfactory program is obtained, all subsequent measurements must be made using the identical program conditions.

From the charts obtained with the standards and samples, read the transmittance at 10.36μ. Convert to absorbance and calculate absorptivities. Draw a base line on the charts from 10.10μ to 10.65μ for acids, from 10.20μ to 10.59μ for methyl esters, and from 10.05μ to 10.67μ for triglycerides (see Fig. 14). Measure the distance from the zero line of the recorder chart to the absorption peak (distance ab), calculate the fractional transmission (bc) as the distance to the absorption peak (ab) divided by the distance to the base line (ac), convert to absorbance, and calculate the "background corrected absorptivity." If the chart paper is calibrated in absorbance, subtract the absorbance at the base line (c) from the absorbance at the 10.3μ maximum (b) to get the absorbance of the sample.

Calculate percent *trans* isomer as elaidic acid, methyl elaidate, or trielaidin as follows:



Infrared absorption of *trans* unsaturation in esters. (Fig. 14)

$$\text{percent } trans = \frac{\text{absorptivity } (A/bc) \text{ of sample} \times 100}{\text{absorptivity of elaidic acid, methyl elaidate, or trielaidin}}$$

Where A = absorbance = $\log 100/T$
b = internal cell length in cm
c = concentration of sample in g/liter
T = percent transmission

By Gas Chromatography

Equipment: Any gas chromatograph equipped to use capillary columns. Stainless steel capillary columns (length 50m, i.d. 0.254mm) coated with an 8 percent w/v solution of EGSS-X (Applied Science Laboratories, Inc.) in methylene chloride. A polar wetting agent such as Alkaterge T (an amine surfactant) is added to the packing solution to give a concentration of 0.2 percent. Suitable prepacked columns also can be purchased from Perkin Elmer Company.

Conditions for analysis: These are the conditions used by Lavoue and Bezard (*13*), who employed a Barber Colman Model 10 chromatograph equipped with a Sr^{90} ionization detector. Suitable operating conditions for other instruments and detectors must be determined by individual investigators.

Column temperature: 186° to 187°C
Flow rate at column outlet: 0.2 to 0.3 ml/min.
Injector temperature: 300°C
Carrier gas pressure at inlet to injector: 8 to 10 psi
"Split" ratio: about 1:100 (The injected sample is split into two streams so that only a portion of the sample enters the column.)
Amount of solution injected: about 2μl
Rate of back flush (scavenger): 60 ml/min.

The authors found that in addition they had to employ a shunt (flow rate 8 ml/min.) to reduce the dead volume between the column outlet and the inlet to the detector. This seems to be necessary when using capillary columns with an argon ionization detector that has a large internal volume relative to the volume eluted at the column outlet. This problem is not encountered with a flame ionization detector.

The detector temperature was 220°C and the carrier gas was argon.

Using this set of conditions, the authors were able to determine efficiently the composition of mixtures of methyl esters of oleic, elaidic, 9-*trans*-12-*trans*, and 9-*cis*-12-*cis* linoleic acids.

The one disadvantage of this procedure is the use of the polar column packing, which has under these conditions a very short life (5 to 10 days). Separation of many *cis* and *trans* isomers can be achieved using a nonpolar column packing such as an Apiezon grease. If suitable conditions for separation can be found using a nonpolar column then it is clearly wiser to use nonpolar packings.

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