H.I.V. of the *Sterculia* oil shows that its cyclopropenoid structure is readily saturated without substantial ring-opening. As noted for epoxides and α-hydroxy conjugated dienes, other investigators (40) have also found hydrogenolysis of functional groups when cyclopropenoid acids are reduced under usual conditions involving longer times at atmospheric pressure in the presence of Adams catalyst.

Most samples reached a definite pressure end point in 1-5 min in our application of the Brown procedure, dependent in part on experimental conditions such as rate of stirring. Occasionally, however, the end point was not so well defined and the reaction tapered off toward the end, with 15-20 min required until there is no further dropwise introduction from the buret. Although further experience with this analytical method is needed for complete evaluation of its potential, we are impressed with the breadth of its applicability, the speed with which an answer may be obtained on a sample of oil, and the precision and accuracy that can be achieved if suitable care is taken in the determination.

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Determination of Polar Lipids: Quantitative Column and Thin-Layer Chromatography

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Abstract

The structures of the polar lipid classes of plants and animals are presented, their nomenclature discussed, and suggestions are presented for clarification of nomenclature. The three general types of quantitative chromatographic procedures (column chromatography, thin-layer chromatography, and combinations of column and thin-layer chromatography) available for polar lipids are reviewed and a new quantitative two-dimensional thin-layer chromatographic procedure is presented. Useful quantitative procedures employing columns of cellulose, silicic acid, silicic acid mixed with silicate, magnesium silicate, and ion exchange celluloses are presented. New findings with diethylaminoethyl cellulose columns are described. New quantitative procedures employing silicic acid, magnesium silicate, or diethylaminoethyl cellulose column chromatography with quantitative thin-layer chromatography are described.

THE PURPOSE OF THE present communication is to review the general classes of polar lipids and their nomenclature, and to present some of the chromatographic techniques available for their determination. Since the nomenclature of polar lipids is unsatisfactory in several respects, this subject is considered in detail. A new quantitative two-dimensional thin-layer chromatographic procedure is presented and two new procedures employing column chromatography (silicic acid or magnesium silicate) with quantitative thin-layer chromatography (TLC) are presented.

Lipid Classes and Nomenclature

Glycerol Lipids

There are two general groups of polar lipids: the glycerol lipids and the sphingolipids. Each of these main groups can be subdivided into subgroups: phospholipids and lipids without phosphorus. Figures 1–17 show schematically some of the classes of polar lipids. The glycerol lipids are shown in Figures 1–10. Phosphatidic acid, phosphatidyl glycerol, and diphos-

phatidyl glycerol (cardiolipin) shown in Figures 1–3 form an important series of acidic lipids of increasing complexity by addition of glycerol and/or phosphatidic acid units. Phosphatidic acid, a phosphorylated diglyceride, is the parent substance of the glycerol phospholipids and is believed to be an important intermediate in the biosynthesis of phospholipids, although it generally does not occur to any appreciable extent in animal tissues. Phosphatidyl glycerol occurs to a small extent in some animal tissues and is present in much larger amts in some plant tissues. Diphosphatidyl glycerol was originally isolated from heart muscle and named cardiolipin. It has since been found to occur widely in plants and microorganisms and the name diphosphatidyl glycerol is therefore most appropriate, although the term cardiolipin is still used. Phosphatidyl choline (lecithin), phosphatidyl ethanolamine, and phosphatidyl serine (Figs. 4–6) are well-known and widely occurring glycerol phospholipid classes. The well-established term lecithin has not been completely replaced by the more systematic name phosphatidyl choline.

There are at least two relatively well-characterized phosphoinositides. Phosphatidyl inositol (Fig. 7) is the most abundant. It occurs widely in plant and animal tissues and has the structure shown in Fig. 7. Phosphatidyl inositol diphosphate, the triphosphoinositide of brain (Fig. 8), differs from phosphatidyl inositol in having two additional ester-linked phosphoric acid groups. A diphosphoinositide has occasionally been reported, but does not appear to have been isolated and demonstrated to be a native constituent of a tissue.

Recently galactosylglycerides have been isolated and characterized and shown to be important constituents of plants (1). The simplest of these is a monogalactosyldiglyceride (Fig. 9). A digalactosyldiglyceride containing two galactose molecules linked to a diglyceride unit has also been reported. There are interesting plant lipid counterparts to the cerebrosides characteristic of nervous tissue.

Plants have been shown to contain a sulfolipid (Fig. 10) that has a sulfonic acid group (2) in contrast to the sulfate ester glycolipid (cerebroside sulfate or sulfatide) of brain (Fig. 14). Rouser et al. (3) demonstrated the presence of a direct carbon to phosphorus bond in a sphingolipid of the sea anemone (Fig. 16) and thus C-S and C-P bonds as well as the corresponding C-O-S and C-O-P bonds have been established as occurring in nature in major lipid classes. The first reports of the C-P bond occurring in nature were in crude lipid extracts and as a water-soluble free amino acid (4,5).

The nomenclature of polar lipids is far from uniform and has undergone a series of changes that have not been uniformly accepted. Name changes were proposed as older terms became less precise in the light of new knowledge. The situation is well illustrated by the changes in nomenclature that have been proposed for the diacyl glycerylphosphorylethanolamines (Fig. 5). Originally this lipid class was called cephalin, but in time it became clear that diacyl glycerylphosphorylethanolamine was not the only class of polar lipid in the usual cephalin fraction obtained by precipitation with alcohol. Folch (6) identified diacyl glycerylphosphorylserine and inositol phosphatides in the alcohol insoluble fraction, then generally called cephalin, and suggested that the term cephalin be used to designate the alcohol insoluble fraction and not the glycerylphosphorylethanolamine-based lipids. Folch proposed the term phosphatidyl ethanolamine for the pure glycerylphosphorylethanolamine based lipid from the brain and suggested that corresponding names for the serine, choline, and inositol lipids be used. The terms phosphatidyl serine and phosphatidyl inositol are widely accepted since these lipid classes had not been named before, but phosphatidyl choline has not been as generally accepted in place of the well-established name lecithin. We still frequently use the term lecithin because it is short, well established, and well defined.

When Folch proposed the name phosphatidyl ethanolamine, the nature of the "ethanolamine plasmalogen" was not defined and in fact the Folch preparation of "phosphatidyl ethanolamine" was largely plasmalogen. Subsequently it was proposed that the term phosphatidyl ethanolamine be reserved for the diacyl glycerylphosphorylethanolamines and "phosphatidal" for the corresponding monoacyl derivatives with an α,β-unsaturated ether linked carbon chain replacing the second acyl group. This proposal has not met with uniform acceptance. (It is difficult to distinguish the terms phosphatidyl and phosphatidal, particularly when spoken.) We prefer to use "phosphatidyl" as a generic term for all glycerylphosphorylethanolamine lipids. "Phosphatidyl" thus denotes a glycerol phospholipid. In the past we have distinguished the different glycerylphosphorylethanolamine lipids as diacyl phosphatidyl ethanolamine and phosphatidyl ethanolamine plasmalogen (or more simply ethanolamine plasmalogen). Since glyceryl ethers have recently been isolated from hydrolysates of ethanolamine lipids (7) and since the vinyl ether linkage has become well established for the plasmalogens (8), we now prefer to designate these forms as diacyl phosphatidyl ethanolamine, vinyl-ether phosphatidyl ethanolamine (plasmalogen), and alkoxy phosphatidyl ethanolamine (glyceryl ether form).

The more general term plasmalogen is still conveniently used when referring to all lipid classes together that possess a vinyl ether linkage. Plasmalogen forms of glycerides have been reported.

Evidence is now available that indicates the natural occurrence of still another form of phosphatidyl ethanolamine. We have isolated a lipid class that gives ethanolamine on acid hydrolysis and is chromatographically indistinguishable from the other forms of phosphatidyl ethanolamine on silicic acid columns or by silicic acid impregnated paper and TLC. This new form is separable from the other three forms by column chromatography on diethylaminoethyl (DEAE) cellulose. We have established that the diacyl, vinyl ether, and alkoxy forms of phosphatidyl ethanolamine are eluted together from DEAE with chloroform/methanol mixtures; the new form is not eluted with chloroform/methanol mixtures, but it is eluted with acidic or basic solvents and in part with methanol. Since this behavior has been observed for some of the oxidation products of phosphatidyl ethanolamine, we believe this type to be a series of naturally occurring autoxidation products. We have encountered this new type of phosphatidyl ethanolamine in beef heart, beef heart mitochondria, and human brain. We will refer to this new form as altered phosphatidyl ethanolamine until the chemical differences have been defined.

The present proposal appears to be the first to include all of the different forms of glycerol lipids in a convenient, simple, and meaningful group of names. Phosphatidyl choline (lecithin) and other glycerol

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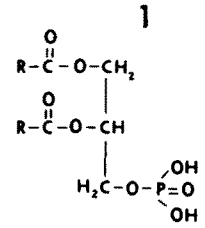

PHOSPHATIDIC ACID

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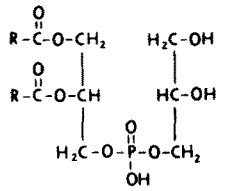

PHOSPHATIDYL GLYCEROL

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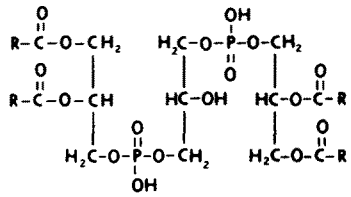

DIPHOSPHATIDYL GLYCEROL

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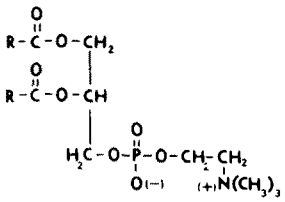

PHOSPHATIDYL CHOLINE

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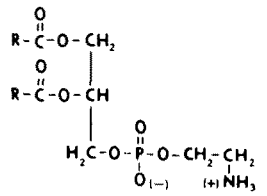

PHOSPHATIDYL ETHANOLAMINE

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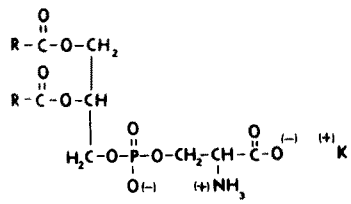

PHOSPHATIDYL SERINE

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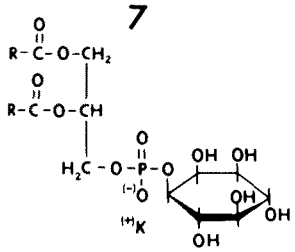

PHOSPHATIDYL INOSITOL

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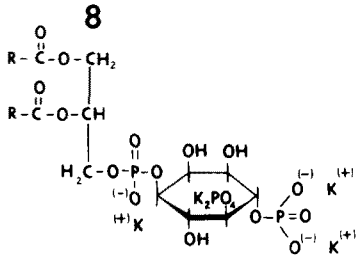

PHOSPHATIDYL INOSITOL DIPHOSPHATE

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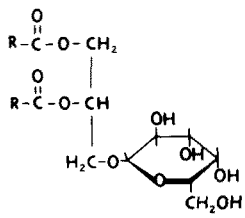

GALACTOSYLGLYCERIDE

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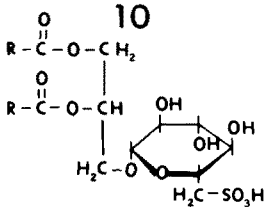

SULFOLIPID

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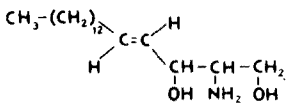

SPHINGOSINE

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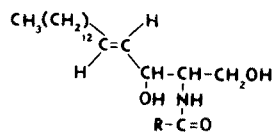

CERAMIDE

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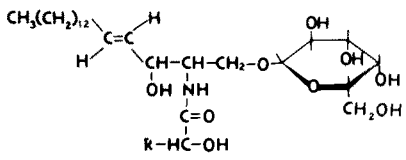

CEREBROSIDE

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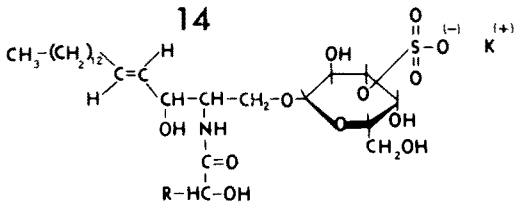

A SULFATIDE

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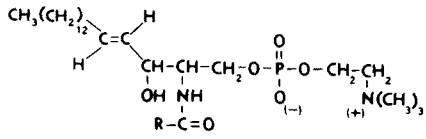

SPHINGOMYELIN

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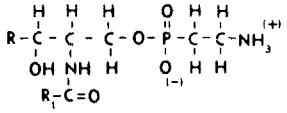
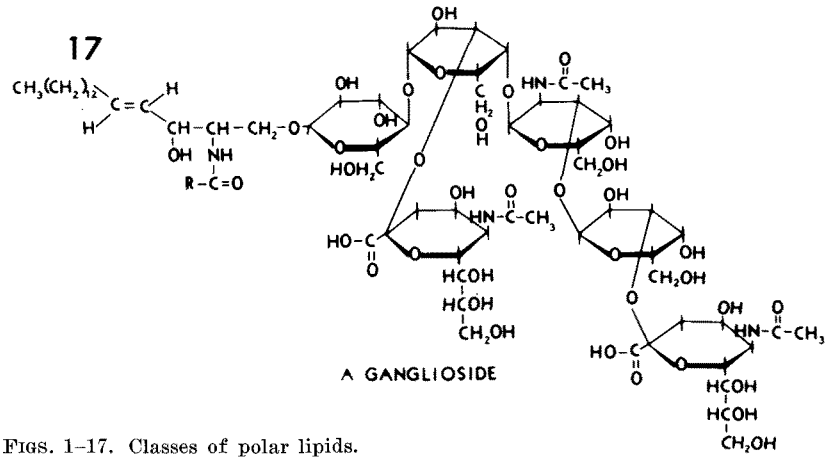


CERAMIDE AMINOETHYLPHOSPHONATE

17



A GANGLIOSIDE

FIGS. 1–17. Classes of polar lipids.

phospholipids can be designated as diacyl, etc., in a similar manner.

Another system has been proposed with which the general term ethanolamine phosphoglyceride (also choline, serine, inositol, etc.) is used, and phosphatidyl and phosphatidal are used specifically for diacyl, and plasmalogen forms. This seems less appropriate than the system we recommend because, although "ethanolamine phosphoglyceride" is not ambiguous, "phosphatidyl" does not have a uniformly accepted meaning (and requires definition) and no provision is made for all forms of ethanolamine phosphoglycerides.

Sphingolipids

Sphingolipids contain a long chain base, either sphingosine (Fig. 11) or a related substance, such as a fatty acid amide. It is clear that nitrogenous bases other than sphingosine are present in plant and animal tissues. Plants have a related base called phytosphingosine (9), nervous tissue contains dihydrosphingosine, and a C_{20} analog of sphingosine has been demonstrated (10). It is probable that still other related bases occur, at least in minor amts, in various plant and animal tissues. The simplest sphingolipid is ceramide (Fig. 12) and it may be considered as the parent substance of the sphingolipids. The other sphingolipids are progressively more complex derivatives of ceramide. Ceramide has been isolated and characterized from brain (3,11). Yeast cerebrin is also a ceramide (12). The cerebrosides (Fig. 13) that occur abundantly in nervous tissue have a carbohydrate, usually galactose, attached to the primary hydroxyl group of ceramide, and may contain either a 2-hydroxy fatty acid or a nonhydroxy fatty acid. The sulfatides (Fig. 14), characteristically occurring in relatively large amts in nervous tissue, have a sulfate (ester) linkage to the sugar residue of a cerebroside. Sulfatides have been isolated with 2-hydroxy fatty acids and nonhydroxy fatty acids. The most complex of the sphingolipids is the lipid class known broadly as the gangliosides. There are several types of gangliosides characterized by their content of sialic acid (N-acyl neuraminic acid). In addition, the gangliosides contain several carbohydrate residues. It seems probable that there are at least three general classes of gangliosides occurring in brain: mono-, di-, and trisialogangliosides containing one, two, or three sialic acid residues (13). A disialoganglioside (14) is shown in Figure 17. The name "hematoside" has been proposed for the sialic acid-containing lipids that do not contain an amino sugar (15). At the present time these substances are usually referred to as gangliosides, but some differentiation of the different types of sialic acid-containing lipids seems desirable and will probably be used in the near future.

Ceramide aminoethylphosphonate (Fig. 16) was isolated recently from the sea anemone (3). It is the first example of a ninhydrin-positive sphingolipid as well as the first phospholipid to be isolated that has a direct C-P bond. In naming this new sphingolipid we chose a name we felt would be the clearest from the chemical standpoint. Following the same general nomenclature procedure, cerebrosides would be named ceramide monogalactosides (or glucosides); sphingomyelin, ceramide phosphorylcholine; sulfatide, ceramide monogalactoside sulfate, etc. This nomenclature is presently in use when it is necessary to refer to substances intermediate between cerebrosides and gangliosides. Thus Rapport's cytolipin H, a member of a class of lipids he has termed cytosides (8), can be referred to as a ceramide dihexoside. Other new lipid classes with three or four carbohydrate residues (16) can be named in this way. The name "globoside" has been proposed for those lipids containing the ceramide unit, sugars, and hexosamine, but no sialic acid (15).

A number of additional polar lipid classes have been encountered in microorganisms. Some of these are derivatives of lipid classes discussed above, e.g., amino acid esters of phosphatidyl glycerol and derivatives of phosphatidyl inositol containing several additional carbohydrate residues linked to inositol.

It is apparent that lipids are important in the structures of plants, animals, and microorganisms and that certain definite types of lipids are present in all forms of life. It appears that a certain molecular shape and type of polar group may be required for a particular function and that a plant may have a lipid class different from an analogous lipid class in animals but meeting the same general requirements. Thus plants contain galactosyldiglycerides and sulfonated derivatives of these, while animal tissues contain cerebrosides and sulfated cerebrosides (sulfatides). In contrast, phosphatidyl choline and phosphatidyl ethanolamine occur widely in both plants and animals, but plants containing characteristically the diacyl forms while animals have diacyl, vinyl ether (plasmalogen) and alkoxy (glyceryl ether) forms. Diphosphatidyl glycerol is important in both plants and animals. Amino acid esters of phosphatidyl glycerol occur in microorganisms and probably also in some animal tissues. This lipid class is clearly related with respect to types of polar functional groups to phosphatidyl ethanolamine and phosphatidyl serine (phosphate, amino, and carboxy groups present). A ganglioside type structure is also evident in the bacterial phosphatidyl inositol derivative containing a number of carbohydrate residues.

Column Chromatographic Techniques

The three general types of chromatographic procedures available for the fractionation of complex lipid mixtures are: (1) column chromatography, (2) TLC, and (3) combinations of column chromatography with TLC. Each of these has certain advantages and limitations. Quantitative determination of the various polar lipid classes from almost any source can be accomplished with these procedures provided the basic principles of the methods are understood and the methods are carefully applied. The basic features of the methods are described here so that the chromatographic fractionation of a particular lipid mixture may be performed in an intelligent fashion.

Column chromatographic techniques are the most reliable and basic procedures presently available for the isolation of lipid classes for quantitative class determination and for the determination of the fatty acid composition of each lipid class. Many materials may be used for column chromatography. Initially aluminum oxide (alumina), silicic acid (silica gel), and cellulose were used. Alumina is of limited use since changes can be produced by adsorption onto this very strong adsorbent. One of the most significant changes brought about by alumina and pertinent to the present discussion is the formation of lysolecithin from lecithin. Despite these difficulties, some investigators have attempted to use alumina for quantitative work. We will not consider these procedures. A strong adsorbent like alumina is useful for separations of nonpolar lipids, particularly hydrocarbons.

Cellulose Columns

Cellulose columns have been described as useful for the separation of water soluble nonlipid materials from lipids prior to carrying out other chromatographic separations and the separation of the water soluble lipids, the gangliosides, from other lipids (11). The gel filtration type cellulose has recently been reported as useful for the separation of water solubles from lipids (17). Removal of water-soluble substances prior to chromatography on other types of columns is desirable because the water-soluble substances may appear in various column fractions. Salts can cause partial elution of some acidic lipid from DEAE cellulose columns with chloroform/methanol or methanol which would not otherwise be effective eluting solvents (chloroform/methanol/salt or methanol/salt mixtures are very powerful eluting solvents for acidic lipids bound to DEAE columns).

A number of difficulties are encountered with cellulose columns. These are partition chromatographic columns and separations are carried out using chloroform/methanol/water or chloroform/ethanol/water mixtures that tend to produce channels in the column and allow admixture of fractions.

We have not been able to reproduce regularly column procedures similar to that of Svennerholm (18), but we have found another procedure to be useful and rather reproducible (19). A slurry of standard grade Whatman cellulose powder is prepared in methanol/water 9/1 and packed into a chromatography tube to a height of 20 cm. The bed is washed with 5 bed vols each of methanol/water 1/1, methanol/water 9/1, and chloroform/methanol 1/1. Chloroform/methanol 9/1 saturated with water is then passed through the bed until one phase only appears from the column. Lipids are applied in chloroform/methanol 9/1 saturated with water, and the same solvent (about 8 bed vols) is used to elute the major lipid classes (except gangliosides) together. Methanol/water 9/1 is then used to elute nonlipids and gangliosides. Repeated attempts to obtain quantitative separation of gangliosides from the water-soluble nonlipids have failed. Invariably, some nonlipid is eluted with the gangliosides. We recommend this cellulose column procedure for the separation of water solubles from lipids other than gangliosides in a reproducible manner.

The low solubility of triglycerides of saturated fatty acids in solvent mixtures saturated with water can present difficulties. Some triglyceride may tail back into the water-soluble fraction unless very large elution vols are used and traces of sulfatide and phosphatidyl serine may be present in the water-soluble fraction.

Silicic Acid Column Chromatography

Silicic acid, sometimes called silica gel, has been the most widely used substance for column chromatography of lipids. Silicic acid is a relatively mild adsorbent from which nearly all polar lipids can be eluted without alteration. Silicic acid has been used to separate the so-called neutral lipids from the phospholipids and other polar lipid classes. Effective use of silicic acid columns requires an understanding of the underlying principles upon which the separations are based. Silicic acid preparations may differ in a number of ways. They differ in particle size which is clearly related to their total capacity to bind lipids. Larger particle sizes are usually preferred for column chromatography since proper flow rates are difficult to obtain with fine particles. Since resolution in column chromatography is greatly improved when particles are of small, uniform size, it seems probable that more use will be made of fine particles with a pressure system to produce an adequate flow rate.

The most important variations in silicic acid preparations other than particle size and uniformity are concerned with the presence of varying amounts of water, silicate, and other salts. Ionic lipid and silicic acid undergo ion exchange reactions (3,11). In this process the dissociation of acid groups of acidic lipids is depressed and in this less ionized state the lipids are less polar and more readily eluted. Fatty acids are readily eluted from silicic acid with mixtures of diethyl ether in hexane, i.e., as neutral lipids. Elution of fatty acids from magnesium silicate columns where fatty acid is present in the ionized state requires the more polar chloroform/methanol mixtures. This illustrates the marked variations in elution characteristics dependent upon the ionic state of the lipid.

Acidity of silicic acid preparations decreases as silicate content is increased. Under these conditions the acidic groups of ionic lipids are more highly ionized. The result is that acidic lipids are less readily eluted from silicic acid columns containing silicate and changes in the relative order of elution of acidic lipids with respect to nonacidic lipids can be observed at high silicate contents. Rouser at al. (20) made use of this fact for the separation of phosphatidyl ethanolamine from phosphatidyl serine using silicic acid columns treated with aqueous ammonia.

Some silicate is usually present in silicic acid preparations because the mixture of sodium silicate and mineral acid used to prepare silicic acid is washed by decantation. The salt formed in the initial reaction will ion exchange with silicic acid to produce some silicate sites under these conditions. If the silicic acid is washed on a filter first with acid and then water, all salt may be removed and a very pure silicic acid produced. Such preparations are sufficiently acidic to cause partial degradation of the vinyl ether (plasmalogen) forms of phospholipids and are thus of limited use.

Residual salt, e.g. sodium chloride, in silicic acid preparations can give rise to traces of mineral acid with resultant changes in elution characteristics of ionic lipids and some degradation of plasmalogens. This salt effect has been used to advantage in silicic acid impregnated paper chromatography (21).

The elution of "neutral" or less polar lipids as a group from silicic acid with chloroform (usually containing 0.2–0.7% alcohol as stabilizer) is useful and very popular for the separation of "neutral" lipids (hydrocarbons, glycerides, sterols, sterol esters, etc.) from polar lipids. Even when only traces of polar lipids are present (as in processed fats and oils, milk fat, or adipose tissue lipids), neutral lipids can be freed of the more polar lipids by this method and very large loads can be applied to obtain an adequate quantity of polar lipid for characterization. The polar lipids are eluted together with methanol or separated into groups by stepwise increases in the concn of methanol in chloroform. The exact methanol concn for rapid elution of different lipid classes depend upon the water content of the system. The presence of a small amount of water may be associated with increased binding of lipids (3,11,21), particularly in the presence of silicate. As the water content of the system is increased even more, lipids are less firmly bound and elution with lower methanol concns is possible.

Variations noted above for silicic acid preparations must be considered if reproducible results are to be obtained. The variations in elution characteristics with different preparations can be used to increase the usefulness of silicic acid columns for special separations. The greatest defect of silicic acid columns for the separation of complex lipid mixtures into pure individual lipid classes is the elution of acidic lipids along with nonacidic lipids (3,11). A complex mixture must therefore be simplified prior to application to silicic acid or mixtures from silicic acid columns must be separated on other columns.

Silicic Acid-Silicate Columns

Rouser et al. (11,20) introduced the use of silicic acid columns containing silicate and water for special separations. Silicic acid-silicate columns were applied initially for the separation of phosphatidyl ethanolamine from phosphatidyl serine of beef brain, and subsequently used for the separation of lecithin from sphingomyelin. Elution from silicic acid-silicate columns is accomplished with chloroform/methanol or chloroform/methanol/water mixtures. Silicic acid-silicate columns are prepared by one of two procedures. A silicic acid column may be prepared and a mixture of chloroform/methanol/water containing base may be passed through the bed to introduce silicate, primarily at the top of the column. This is the procedure utilized originally for the separation of phosphatidyl ethanolamine and phosphatidyl serine (20). The alternative approach is to mix the silicic acid uniformly with aqueous base to introduce silicate throughout the bed as utilized for the separation of lecithin from sphingomyelin (3,11).

Silicic acid-silicate columns can be used for separation of the neutral lipids from the phospholipids and other polar lipids by applying the mixture to the column in chloroform and elution of neutral lipids with chloroform. Fatty acids are retained along with other polar lipids since they remain as salts. In this respect silicic acid-silicate columns are similar to magnesium silicate columns. The various polar lipid classes are eluted from silicic acid-silicate columns with increasing concns of methanol in chloroform or water in chloroform/methanol (3,11).

Magnesium Silicate Columns

Radin (22) introduced the use of magnesium silicate columns for the recovery of cerebrosides from brain lipids and other investigators have used magnesium silicate for various separations including the separation of neutral lipids from polar lipids, the separation of individual neutral lipid classes (23), and the separation of individual polar lipid classes (3,11). Our own experience with magnesium silicate (Florisil) has indicated that it is useful under carefully controlled conditions for the quantitative separation of polar lipids from neutral lipids. Magnesium silicate is a much stronger adsorbent than silicic acid. When magnesium silicate is heat-activated and maintained in a thoroughly dry state by using solvents dehydrated with 2,2-dimethoxypropane or a molecular sieve, it has a very high binding capacity for lipids and is suitable for the separation of neutral lipids, ceramide, cerebrosides, sulfatides, galactosylglycerides, and sulfolipids (3,11). The presence of even a small amt of water allows the passage of small amts of phospholipids into very early fractions from a column; i.e., fraction overlap develops.

Magnesium silicate columns have certain advantages for recovery of neutral lipids. A commercial fat or oil sample or other mixtures very low in polar lipids can be completely freed of the polar lipid components by elution of neutral lipids (glycerides, sterol esters, sterols) with chloroform and very large loads can be applied to the column. Polar lipids including fatty acids are retained by the column.

Although methanol elutes all lipids from silicic acid columns, it does not elute all lipid classes from dry magnesium silicate. All polar lipids can be eluted with mixtures of chloroform and methanol containing water, but adsorbent appears in the column effluent. Selective elutions from magnesium silicate are possible. Chloroform/methanol 95/5 can be used for the elution of ceramides, and chloroform/methanol 2/1 can be used for the elution of cerebrosides and sulfatides (animal tissues) or galactosylglycerides and sulfolipids (plant lipids). These elutions can be accomplished without contamination from phospholipids if the column is maintained in a very dry state. The magnesium silicate columns are thus useful for certain polar lipid classes only.

Diethylaminoethyl (DEAE) Cellulose Columns

Since its introduction (24), DEAE cellulose column chromatography has been used extensively for the separation of polar lipids. DEAE columns were developed when it was determined that silicic acid column chromatography was not adequate for the separation of the various polar lipid classes because some acidic lipids are eluted with nonacidic lipids from silicic acid columns. DEAE columns can be used to separate acidic lipids as a group from nonacidic lipids (3,11). Since the elution characteristics of DEAE columns have been described extensively in a recent communication (3), the many elution details will not be repeated here. It is important to realize, however, that acidic lipids are not eluted from DEAE with chloroform, chloroform/methanol mixtures, or methanol under proper conditions. Acidic lipids can be eluted as a group with acidic or basic solvents with or without addition of a salt such as ammonium acetate. Many acidic lipid classes can be eluted in pure form from DEAE columns with appropriate solvents.

We have improved the preparation and elution of DEAE columns since the last communication. One of the most important new developments is the elution of the neutral lipids (glycerides, sterols, and related nonionic substances of similar polarity) free of polar lipids (including fatty acids). This separation is carried out conveniently by preparing a DEAE acetate column in chloroform. The lipid mixture is applied to the column in chloroform and chloroform (at least 5 column vols) is used to elute neutral lipids, followed by various mixtures of chloroform and methanol for nonacidic ionic lipids and the highly polar nonionic lipids (cerebrosides, galactosylglycerides), and finally elution of acidic lipids with acidic or basic solvents.

Initially we were unimpressed with the use of DEAE columns for the separation of neutral lipids by elution with chloroform because some of the more polar lipids appeared along with the neutral lipids. This behavior was traced to the formation of small channels (holes) in the column produced by improper changing of solvents. DEAE cellulose columns (as well as other types of cellulose columns) may channel when treated with solvents containing relatively large amts of water or when a very abrupt solvent change such as from methanol to chloroform/methanol 9/1

or chloroform is made. When the solvent sequence methanol, chloroform/methanol 1/1, then chloroform is used, channeling does not take place and good separations can be obtained.

Because of the importance of channeling we now advise a simple test procedure for demonstrating the presence or absence of channels. This test can easily be carried out with cholesterol or a glyceride. The column is placed in chloroform (or chloroform/methanol 9/1), a small amt of recently recrystallized cholesterol dissolved in chloroform (or chloroform/methanol 9/1) is applied to the column, and the solvent used for application to the column is then used to elute cholesterol. With a 2.5 x 20 cm column (15 gm of DEAE) using 10 ml fractions, cholesterol should appear first in tube 7 or 8 if channeling is absent. If the appearance of cholesterol is earlier than this, channels are present, and if cholesterol appears by fraction 5 or earlier, the column should be repacked. This simple test can be carried out just before application of the sample. If the test shows the column to be satisfactory, cholesterol can be eluted quantitatively and quickly with chloroform or chloroform/methanol 9/1 (ca. 2 column vols) and the sample applied.

Data collected since the last communication have also disclosed some interesting features about the nature of overloading of DEAE columns and the elution characteristics of certain lipid classes that depend upon the relative composition of the lipid mixture applied to the column. Some of the effects of overloading of DEAE columns have already been described (3), but we now have additional examples of special types of overloading. It has been found that the elution of phosphatidyl inositol diphosphate (triphosphoinositide) from brain lipids with a mixture of chloroform/acetic acid 3/1 as previously described (3) represents a special case. This elution is observed only when a relatively large amount of brain lipid or a preparation rich in triphosphoinositide is applied to the column. This appears to be a direct influence of salts and/or other ionic lipids upon the elution properties of triphosphoinositide. When the amt of total lipid, and particularly the amt of triphosphoinositide, is reduced, triphosphoinositide is not eluted with chloroform/acetic acid 3/1. Triphosphoinositide is bound so firmly that the most powerful eluting solvents (acetic acid-ammonium acetate or chloroform/methanol/ammonia/ammonium acetate) are required for elution.

The presence of large amts of gangliosides or free fatty acids is also associated with overloading phenomena at loads that would otherwise be appropriate for the lipid mixture. A marked difference in the elution characteristics of cardiolipin has also been observed depending upon the amt of cardiolipin and the amt of other acidic lipids in the mixture. Thus, while we have been able to elute cardiolipin quite readily with chloroform/methanol 4/1 containing 20 ml per liter of 28% aqueous ammonia with beef liver or beef brain lipid as samples, this solvent is not as effective for cardiolipin of beef heart mitochondrial lipid. Beef heart mitochondria contain a great deal of cardiolipin and very little acidic lipid of other types. It appears that the presence or absence of other acidic lipids determines to some extent the ease with which cardiolipin can be eluted. The load of lipid applied is also important and it must be emphasized that at high loads a portion of a particular acidic lipid class may be eluted with solvents that do not elute the same lipid at all at lower loads.

Because of the differences in elution characteristics with different lipid mixtures, we recommend the following: First, that a cellulose column be used to eliminate the water-soluble nonlipids; Second, that low loads be applied. Optimum resolution on a DEAE column 2.5 x 20 cm prepared using 15 g of ion exchange cellulose as previously described (3) can usually be obtained when from 90 to no more than 200 mg of a lipid mixture is applied where there is a large amt of acidic lipid (as for beef brain white matter or lipids extracted from whole human brain). We have found that pathological human brain specimens may require the cellulose column treatment and very low loads (80–100 mg of total lipid). A great deal of time may be saved by purposely low-loading DEAE columns. Low-loading can give pure lipid classes readily and accurate quantitative values may be obtained for individual lipid classes. It is to be emphasized, however, that this introduces another important feature. In order to measure accurately the lipid fractions by weight, it may be necessary to utilize a microbalance for some of the fractions.

DEAE cellulose columns are particularly useful when used in conjunction with silicic acid and silicic acid-silicate columns for the complete fractionation of complex lipid mixtures (3). They are also quite useful when combined with quantitative TLC (see below).

Other Ion Exchange Celluloses

Various other ion exchange celluloses have been explored in this laboratory for their possible use as column materials for lipid separations. Phosphocellulose has been demonstrated to be a strong adsorbent for polar lipids, particularly in the presence of water, but this has not been utilized routinely because of the problems associated with controlling water content (24). Similar problems exist for sulfoethylcellulose. Triethylaminoethyl (TEAE) cellulose has also been explored to a limited extent (3) and it has been demonstrated that, as expected according to ion exchange theory, phosphatidyl ethanolamine is not eluted from TEAE with methanol or other nonionic polar solvents. Acid, base, or salt must be added to the eluting solvent for elution of phosphatidyl ethanolamine from TEAE in contrast to DEAE cellulose. These observations are of potential value where the more commonly used DEAE or other column types do not give the desired separations. Pure phosphatidyl ethanolamine can not be eluted from DEAE with chloroform/methanol 7/3 from beef liver lipids because of the presence of methanol soluble lipids that are eluted together with phosphatidyl ethanolamine. The mixture of ionic and nonionic lipids eluted from DEAE with chloroform/methanol 7/3 can be passed through a TEAE column. Nonionic substances can be eluted with neutral solvents and phosphatidyl ethanolamine can be eluted with an acidic or basic solvent.

Quantitative Thin Layer Chromatography

General Considerations

TLC is a very popular technique and useful to the lipid chemist. Its chief advantages over paper chromatography are improved resolution and ease of preparation of different layer thicknesses with different adsorbents. Improved resolution results in part from the fact that thin layer plates can be spread with a relatively finely divided adsorbent of relatively uniform particle size. Silicic-acid-impregnated paper can not be controlled in this manner and resolution

suffers because of lack of uniformity of the adsorbent imbedded in the paper. Microscopically the TLC adsorbent can be seen as particles whereas impregnated paper appears to be more of a connected, uneven matrix rather than particles. The role of uniformity of the adsorbent bed is well illustrated by results with a piece of chromatography paper and the same paper finely ground and spread over a glass plate. Resolution on the plate is improved. Various layer thicknesses that are easily controlled in TLC are useful for analytical and preparative work. These wide variations in layer thickness are not obtainable with impregnated paper. Paper is difficult to impregnate heavily.

It has frequently been stated that TLC is always a more rapid technique than impregnated paper chromatography. This is not uniformly true; in fact, it is clear that with many solvent mixtures silicic-acid-impregnated paper systems are very similar in running times to comparable TLC systems (21). Paper can be impregnated very lightly with silicic acid or other adsorbent and extremely fast chromatographic systems can be devised that are even faster than TLC, but these paper chromatographic systems suffer from the fact that only a small amt of lipid can be applied to the low-loaded papers.

Successful use of TLC for quantitative work depends upon the clear recognition of the variable nature of this technique and how the variables can be controlled, the availability of a suitable color reaction for the actual quantitative determination, and a full appreciation of the limitations of the methods. In order to understand the variables in TLC it is necessary to appreciate that, in contrast to the frequently expounded view that TLC is an adsorption process, it is probably largely a partition chromatographic process and every attempt must be made to eliminate strong adsorption properties in order to eliminate spreading of spots. Adsorption chromatography is clearly and readily distinguished by the presence of streaking of spots. The partition process on the other hand gives sharply defined spots. With the ordinary silica gel preparations containing $CaSO_4$ as a binder, only acidic substances including fatty acids, sulfolipids, and the acidic phospholipids show appreciable adsorption properties.

There are two ways to eliminate adsorption (spreading of spots). First, adsorption is greatly decreased by substituting magnesium silicate for $CaSO_4$. We recommend the use of magnesium silicate as a binder for TLC because, while the binder firmly attaches the adsorbent to the glass plate, adsorption effects are minimal. Even with magnesium silicate there is some spreading of spots of acidic lipids. To completely eliminate the spreading, it is necessary to incorporate an acid into the chromatographic system. This depresses the dissociation of the acidic lipids and prevents adsorption.

Variations in Relative Migrations of Polar Lipids

The ease with which varying thicknesses of adsorbent can be spread onto plates makes TLC a versatile technique, but it also presents some interesting problems. Recently we described an interesting but frustrating phenomenon. We observed a great variation in the relative migration of acidic lipids compared to nonacidic lipids (3). This behavior has now been traced to factors affecting the water content of the chromatographic system when chloroform/methanol/water mixtures are used as developing solvents. We will briefly describe the phenomenon and its basis.

For this description it will be assumed that the chromatography chambers (11) are lined on all sides with paper saturated with solvent immediately before insertion of the plates. With these chromatographic conditions and chloroform/methanol/water mixtures as developing solvents, the positions of the acidic lipids relative to nonacidic lipids may change markedly depending upon the water content of the solvent mixture used and the thickness of the adsorbent layer. This effect can be illustrated by decribing the behavior of the sulfatides relative to cerebrosides, phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin. When water is left out of the solvent completely, sulfatide migrates between cerebroside and phosphatidyl ethanolamine. This is observed using chloroform/methanol 2/1 as solvent (silicic acid with 10% magnesium silicate as binder). When water is added, the exact relationship of sulfatide migration to the nonacidic lipids depends upon layer thickness. Using chloroform/methanol/water 65/25/4, a relatively thick layer of adsorbent can be observed to give migration of sulfatide with or slightly behind phosphatidyl ethanolamine, while medium thick adsorbent layers may be observed to give migration of sulfatide either with or slightly ahead of lecithin, and very thin adsorbent layers may show migration of sulfatide between lecithin and sphingomyelin. Despite these marked variations in acidic lipid migration, there is no change in the order of migration of cerebroside, phosphatidyl ethanolamine, phosphatidyl choline, or sphingomyelin and only slight changes in R_f. It is thus possible to vary the position of sulfatide migration from one end of the chromatogram to the other and to change the relative order of migration with respect to cerebrosides, phosphatidyl ethanolamine, lecithin, and sphingomyelin over a wide range.

This behavior is related to the varying uptake of water by the adsorbent during chromatography. Since the amount of water available from the chamber liner and solvent is kept essentially constant in all cases, a thick layer of adsorbent will, over the approximately 1 hr period required to develop a chromatogram, contain less water per unit wt of adsorbent than thinner layers and hence the chromatographic system effectively contains less water (thick layers of adsorbent). As a result, sulfatide migrates with a greater R_f value on thicker adsorbent layers with little or no change in the R_f values of nonacidic lipids. With thinner layers there is relatively more water in the adsorbent ahead of the migrating solvent, and hence more water in the chromatographic system. This results in a somewhat greater binding of the acidic lipid to the stationary phase and sulfatide migration from the point of application is less on the thinner layers. Similar behavior is observed with other acidic lipids and can be used to place these lipids in convenient positions for quantitative determination.

The basis of these differences in migration depending upon water content is to be found in the differences in the strength of binding of acidic and nonacidic lipids to adsorbents that has been encountered in column chromatography. It was found by column chromatography that acidic lipids are more firmly bound to some adsorbents in the presence of water (3,11,20,24). Of course at high water concns this effect is reversed and all of the lipids are more readily

eluted. There is a considerable difference in the strength of binding through water for acidic and nonacidic lipids accounting for the marked differences in the relative migration of the lipids.

Quantitative TLC with the Sulfuric Acid-Potassium Dichromate Reagent

While quantitation of phospholipids by TLC can be accomplished by elution of spots and determination of phosphorus content (25), a more general procedure applicable to all lipid classes is desirable. Such a procedure is available with the use of the sulfuric acid-potassium dichromate reagent described by Privett and Blank (26) and utilized for quantitative TLC by Blank, Schmit, and Privett (27). Quantitation is accomplished by densitometry. Privett and Blank demonstrated their ingenious use of the charring reagent for quantitative TLC by densitometry to us and we have found it to be a general and useful procedure. The usefulness of this charring reaction for quantitative TLC has now been extended by a careful study of the nature of the charring reaction.

As noted by Privett and Blank, the extent of charring is related to the carbon content of the lipid, and under suitable conditions the charring reaction is a means for determination of carbon content. These investigators also found that charring was related to the shape of the spot and varied considerably with lipid classes migrating to different positions on the chromatogram. We have studied this phenomenon using plates spread with thin slurries of silica gel with $CaSO_4$ or magnesium silicate (both present at 10% concn) as binder. Thin slurries allow the preparation of extremely even layers of adsorbent and are prepared using 3 ml or slightly more of water per gram adsorbent. When very even layers are spread, it can be observed that, after correction for differences in carbon content, the extent of charring as determined by densitometry using an integrator and recorder is directly proportional to the distance of migration of the particular lipid class. Data obtained using mitochondrial lipids and plotted for the response obtained from known amts of cardiolipin, phosphatidyl ethanolamine, and lecithin are shown in Figure 18. The straight line obtained was the same with the same silica gel preparation containing either $CaSO_4$ or magnesium silicate as binder when the adsorbent containing $CaSO_4$ was spread with 0.01 or 0.02 M NaCl instead of water to prevent spreading of the cardiolipin spot.

This direct relationship between the distance travelled and the increase in charring is related to the fact that spots spread during chromatography and that spread increases with increase in distance migrated. This suggested that one of the determining factors in the charring reaction is the amt of lipid in contact with a given amt of silicic acid. Thus when the silicic acid-lipid ratio is high, greater charring takes place, while a decrease in the ratio is related to lower charring intensities.

These results are obtained when the reagent is sprayed on the plate lightly as a fine mist. Charring is a surface reaction that is dependent upon the binding of lipid to silicic acid. Silicic acid appears to enter into the reaction and to be essential since with pure magnesium silicate as adsorbent no charring takes place with the sulfuric acid-potassium dichromate reagent. Similarly with neutral aluminum oxide, only a weak yellowish-brown charring reaction is obtained. These observations indicate that an acidic adsorbent is required for this type of charring reaction to take place.

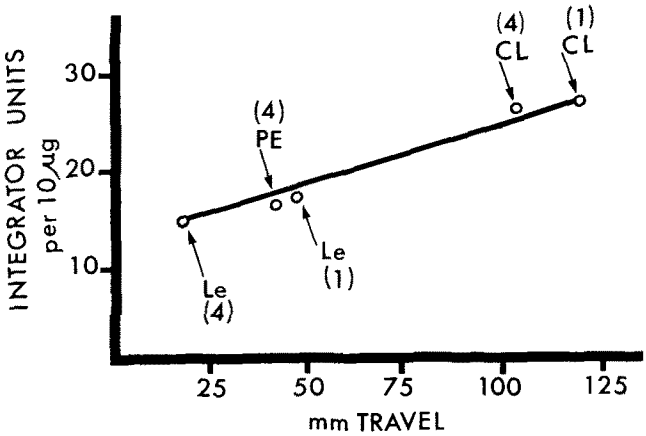


FIG. 18. Plot of extent of charring expressed as integrator units (converted to 70% carbon content for each lipid class) vs. extent of migration (millimeters travelled) by TLC for cardiolipin (CL), lecithin (Le), and phosphatidyl ethanolamine (PE). The straight-line relationship was obtained using mixtures of silicic acid and magnesium silicate or calcium sulfate (both used in the ratio of silicic acid to binder of 9/1 by wt). The numbers in parenthesis indicate the number of determinations averaged for each point. The single determinations for cardiolipin and lecithin were from chromatograms using calcium sulfate as binder while the four determinations averaged for the other points were obtained using magnesium silicate as binder. With chloroform/methanol/water 65/25/4 all of the lipids migrated somewhat further down the plate with calcium sulfate as binder, but the points fell on the line.

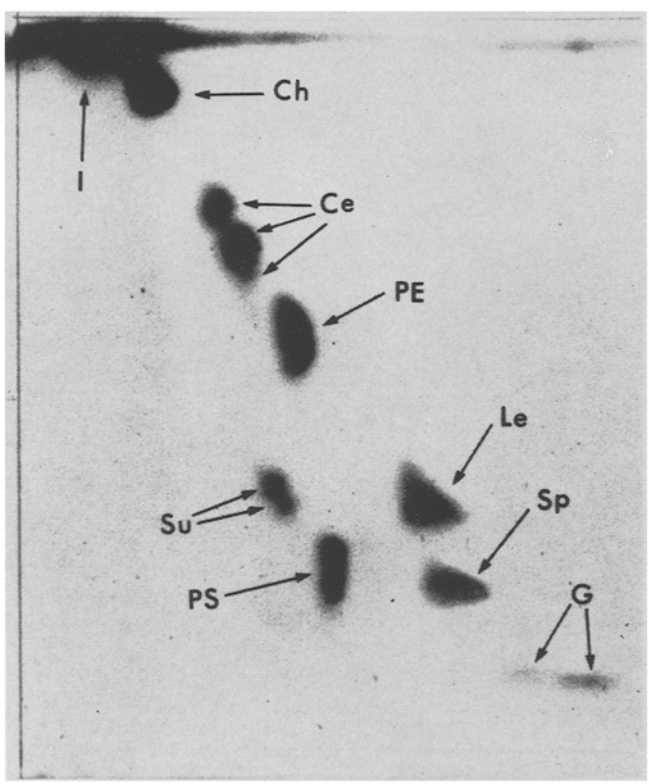


FIG. 19. Two-dimensional TLC using 200 µg of beef brain lipid as sample. The chromatogram was developed in the vertical direction with chloroform/methanol/water 65/25/4, dried for 10 min in air, and developed in the horizontal direction in butanol/acetic acid/water 60/20/20. The chromatogram was then dried in air for several hours, sprayed with the sulfuric acid-potassium dichromate reagent, and heated at 180C for 30 min to develop spots. The substances are I, impurities at the solvent front; Ch, cholesterol; Ce, cerebrosides; PE, phosphatidyl ethanolamine; Le, lecithin; Sp, sphingomyelin; Su, sulfatide; PS, phosphatidyl serine; and G, gangliosides.

TABLE I

Beef Brain Lipids

Lipid Class	Col.	DEAE TLC	TLC 1-D	TLC 2-D
Cholesterol	20.3	20.4	19.0	19.5
Ceramide	0.3	0.4	0.4	0.3
Cerebroside	13.1	13.5	13.7	14.2
Sulfatide	4.0	4.0	3.9	3.5
Lecithin	....	11.3	....	11.0
Sphingomyelin	....	7.9	....	7.5
Phosphatidyl ethanolamine	14.0	14.0	14.7	14.3
Phosphatidyl serine	7.5	7.5	....	7.5
Gangliosides	2.5	2.5	....	....
Phosphatidyl inositol	2.0	2.0		

The values listed under column chromatography were obtained by use of DEAE, silicic acid, silicic acid-silicate, and magnesium silicate columns. The values in the second and third columns obtained by the DEAE-TLC procedure and direct one-dimensional TLC are taken from a paper from Rouser et al. (28). The fourth column obtained by two-dimensional TLC without column chromatography was obtained using chloroform/methanol/water 65/25/4 and butanol/acetic acid/water 60/20/20 as described in the text with beef brain lipid as sample (without prior column chromatography).

By varying the amount of sulfuric acid and potassium dichromate in the reagent we determined that too little potassium dichromate did not give complete charring. Very high concns of potassium dichromate give less intense spots, apparently because the reaction progresses beyond the charring stage with loss of carbon. We have not been able to eliminate the effect of spot spread (the ratio of lipid to silicic acid) by varying sulfuric acid or potassium dichromate concns.

It is apparent that spreading of spots during chromatography must be eliminated if the extent of charring of all lipid classes is to be directly proportional to carbon content only and not influenced by the distance travelled (R_f). Spreading is reduced with magnesium silicate as binder in silicic acid and eliminated with acidic solvents. One-dimensional TLC without spreading of spots is possible with acidic solvents and distance travelled has little effect on the extent of charring.

Quantitative Two-Dimensional TLC

Quantitative two-dimensional TLC with chloroform/methanol/water (65/25/4 or 65/35/5) in the first dimension followed by an acidic solvent (butanol/acetic acid/water 60/20/20 or chloroform/acetone/methanol/acetic acid/water 5/2/1/1/0.5) in the second dimension is useful for quantitative work. With this two-dimensional technique, spreading occurs during chromatography with chloroform/methanol/water mixtures in the first dimension, but the spots are made compact in the acidic system in the second dimension and spot size with the butanol/acetic acid/water solvent is related to the concn of lipid and the carbon content of the lipid class only, and not R_f. The charring is then quite uniform. When very uniform layers of adsorbent are used and spraying is very uniform, standards are not required on each plate. Drying time must be relatively short (ca. 10 min) between development in the first and second dimensions in order to avoid changes in the lipids with production of artifact spots with the second solvent.

Figure 19 shows the results of a two-dimensional TLC run with beef brain lipids and Figure 20 spots from pure lipid standards. From chromatograms of standards it was determined that, when all lipid classes were converted to a common carbon content (usually 100%) for comparison, an almost constant factor is obtained from all lipid classes (all polar lipid classes give nearly the same charring response per microgram of lipid). This property was used to

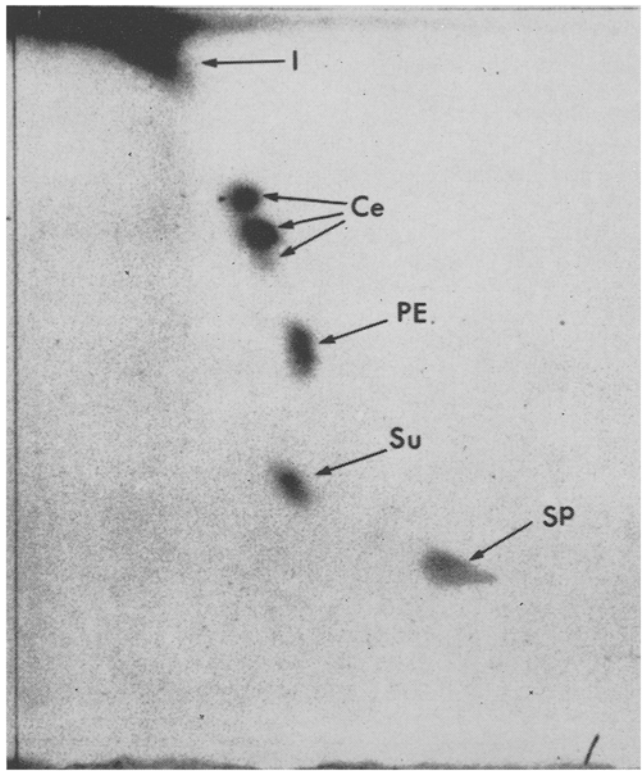


FIG. 20. TLC of a mixture of standard substances prepared from the pure lipid classes isolated by column chromatography. Chromatography and other conditions as specified in the legend for Fig. 19. The chromatogram shows cerebrosides (Ce), phosphatidyl ethanolamine (PE), sulfatide (Su), and sphingomyelin (Sp).

calculate the percentages of the major lipid classes of beef brain shown in Table I. The values agree well with those obtained by column chromatography alone or a combination of DEAE cellulose column chromatography with one-dimensional TLC using lipid standards on the same plate as samples (28).

We submitted a sample of crude soybean oil (supplied by Dr. Donald Wheeler) to a similar analysis. The optimum concn was first determined by one-dimensional TLC. These one-dimensional runs showed that 5 mg of oil was appropriate for two-dimensional TLC. Satisfactory spots of the major lipid classes were obtained without interference from the large amt of neutral lipid. Figure 21 shows a typical two-dimensional TLC of this crude soybean oil sample. The nature of the components was determined as follows. Chromatograms similar to those of Figure 21 were sprayed with ninhydrin and a typical color was obtained in the proper position for phosphatidyl ethanolamine. The reagent specific for phospholipids (29) was positive for the spots marked for phosphatidyl ethanolamine, phosphatidic acid, phosphatidyl inositol, and lecithin. The positions occupied by these lipid classes on the two-dimensional TLC were compatible with these identifications. The extent of charring with a 5 mg sample was then determined. The polar lipid composition of the crude soybean oil was calculated to be phosphatidyl ethanolamine, 0.5%; lecithin 0.4%; phosphatidyl inositol, 0.3%; phosphatidic acid, 0.1%; free fatty acid, 0.4%. No attempt was made to determine the exact amts of the uncharacterized components since the exact carbon content of these lipid classes is not known. Evidently this represents a very rapid, simple, and convenient technique for quality control with many applications. A commercial mixture of soybean polar lipids (Aso-

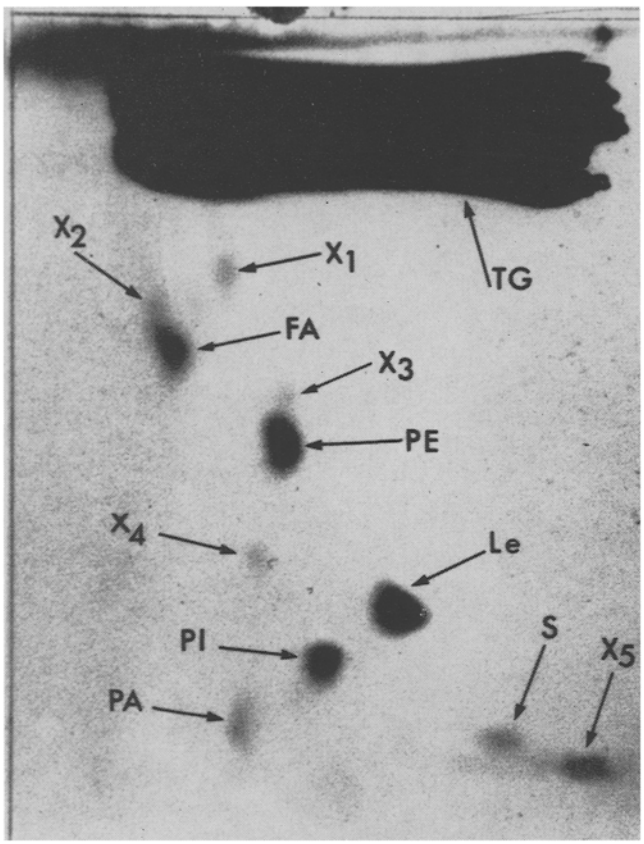


FIG. 21. Two-dimensional TLC of a sample of crude soybean oil. Five milligrams of crude oil was applied to the origin in the lower right hand corner and chromatography carried out as indicated in the legend for Fig. 19. The very small amounts of polar components in the crude oil sample are readily detected without interference from the very large amount of triglyceride shown at the top of the chromatogram. Abbreviations are: TG, triglyceride; unknown substances listed as X_1, X_2, X_3, X_4, and X_5; FA, free fatty acids; PI, phosphatidyl inositol; PA, phosphatidic acid; and S, carbohydrate (probably sucrose). Other abbreviations as in the legend for Fig. 19. See text for the amounts of the various lipid classes determined by quantitative two-dimensional TLC.

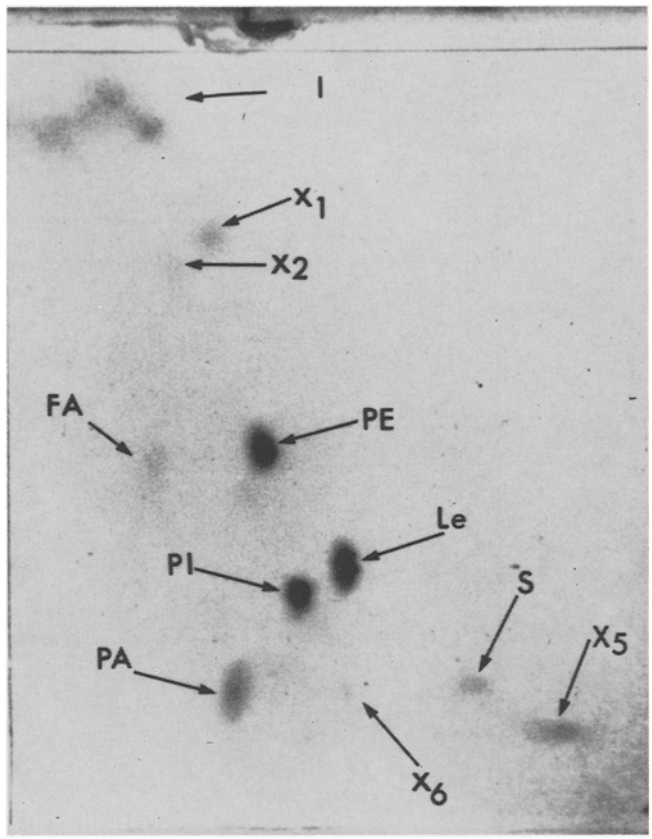


FIG. 22. Two-dimensional TLC of a mixture of commercial soybean phospholipids (Asolectin). A 100 μg sample was applied and developed as indicated in the legend for Fig. 19. Abbreviations as in legends for Figs. 19 and 21.

lectin) was analyzed in a similar fashion. A typical chromatogram is shown in Figure 22. The polar components are similar to the minor components of the crude oil sample.

Standards can be applied on separate plates or to the same chromatogram used for the sample for quantitative two-dimensional TLC. Spotting of two samples on the same plate can be accomplished by spotting one sample above or to the side of the other so that spots of one sample fall near to, but do not overlap with, spots of the sample. An illustration of this technique is shown in Figure 23 where two different applications of beef heart mitochondrial lipid were made to the same plate at the points marked and it is seen that all but the cardiolipin spots were separated. The charring response for the spots of the same lipid class are very similar and this technique can be used to minimize variations.

Limitations of Quantitative TLC

One or two-dimensional quantitative TLC techniques alone are very useful, but there are definite limitations. Minor components may not be detected or may not give a charring response adequate for quantitation, and it is not always possible to increase sample size to obtain a greater reading. At higher concns the major components may spread and obscure the minor components. A great disadvantage of TLC techniques used alone is the uncertainty of identifications based primarily on one or two dimensional TLC migration characteristics. This is a serious disadvantage when working with samples that have not been subjected to other careful isolation and characterization procedures, and we advise great caution in applying these simple quantitative TLC procedures unless information obtained by others means is available.

Some of the uncertainties of identifications of lipid classes based only on TLC migration characteristics can be eliminated by preparation of simple derivatives that are then subjected to TLC. We have used the acetyl and succinyl derivatives for this purpose. These derivatives have been used previously in other studies (30) and are useful in preparative chromatography (e.g., the succinyl derivatives in the separation of lecithin from sphingomyelin). The acetyl derivatives are less polar than the original lipid while the succinyl derivatives are more polar.

Acetylation with minimal side reactions can be accomplished using pyridine/acetic anhydride (2/1) with no more than 10 mg of lipid per milliliter. Heating at 100C for 1 hr gives complete acetylation of beef brain lipids. The solution is evaporated to dryness. The derivatives are then dissolved in an appropriate solvent and subjected to TLC. Comparison is made with standards. Succinylation is a rapid, quantitative reaction when carried out in pyridine with 20-fold calculated excess of succinic anhydride at 100C for 1 hr. After evaporation of solvent, the derivative can be removed for the most part from other reaction products by solution in chloroform.

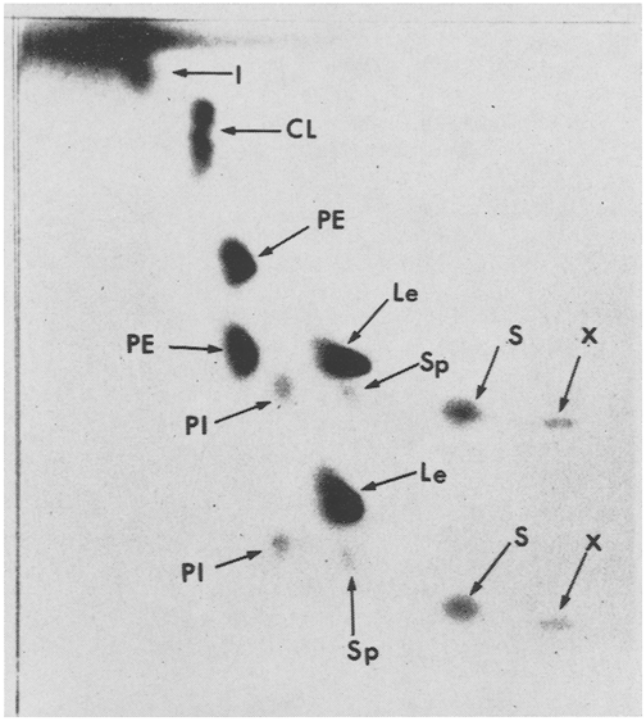


FIG. 23. Two-dimensional chromatogram illustrating the means of spotting two samples on the same plate. Beef heart mitochondrial lipid (100 μg) was spotted at the lower right hand corner and another sample of the same preparation (also 100 μg) was spotted approximately halfway up the plate. Chromatography was then carried out as described in the legend for Fig. 19, and the completed chromatogram was sprayed with the sulfuric acid-potassium dichromate reagent. Note that all spots of the two samples are well separated except those of cardiolipin in the upper left hand corner. Abbreviations are as indicated in the legends for Figs. 19 and 21.

The chloroform solution is then subjected to TLC and identifications made by comparison with standards.

Combinations of Column Chromatography with Quantitative TLC

General Considerations

Precise values can be obtained by quantitative TLC under the proper circumstances. Errors from spot overlap and the inability to detect and determine the minor components of a mixture can be eliminated or reduced by combining column chromatography with TLC. Columns can be used to conc minor components in selected fractions and reduce spot overlap by selecting the most appropriate elution scheme for column chromatography of the particular mixture. We recommend combinations of DEAE cellulose, silicic acid or magnesium silicate column chromatography with quantitative TLC.

DEAE-TLC

Rouser et al. (28) have recently reported a procedure for the determination of polar lipids using a combination of DEAE cellulose column chromatography and quantitative one-dimensional TLC. This is a combination of the DEAE-TLC method of Rouser et al. (3) and the charring-densitometry technique of Blank et al. (27). The method makes use of the numerous modifications that can be made in the elution scheme for DEAE columns. Fractions from DEAE columns are subjected to quantitative TLC with a chloroform/methanol/water mixture or an acidic solvent. Quantitative TLC with standards is carried out by alternately spotting standards and samples for direct comparison on the same plate. Relatively simple elution schemes can be utilized for DEAE columns since the essential features of the column are to prevent overlap of spots by one-dimensional TLC and to conc some of the minor components in special column fractions.

A very useful procedure is to prepare the column in the acetate form in methanol, apply the sample in methanol or some suitable chloroform/methanol mixture in which it is soluble, elute with methanol to obtain all of the nonacidic lipids, followed by elution with chloroform/methanol 4/1 containing 20 ml of 28% (by wt) aqueous ammonia per liter and made 0.01 M with respect to ammonium acetate for the complete elution of acidic lipids. This conveniently divides the sample into nonacidic and acidic lipids (3) and greatly reduces fraction overlap. It may be necessary to use a more complicated elution scheme to facilitate quantitative TLC. Thus a DEAE column may be eluted first with chloroform to separate glycerides, sterols, and related substances (neutral lipids) from the polar lipids, followed by elution of the various polar lipid classes using as many different solvents as desired to reduce the complexity of individual fractions.

Combination of Cellulose and DEAE Columns with Quantitative TLC

A useful method that is currently being used extensively in this laboratory for the quantitative determination of the lipid composition of human brain (normal and pathological) is based on the preliminary use of a cellulose column (see above) for the separation of gangliosides and water soluble nonlipid components from the other major lipid classes. DEAE column chromatography is then used for further separation and the components of mixed fractions are determined by quantitative TLC.

Silicic Acid Column Chromatography and TLC

A combination of silicic acid column chromatography and quantitative TLC is frequently advantageous. We use several systems for brain lipids that can be applied with minor modifications to other lipid mixtures. Relatively simple elution schemes are required for columns. A useful elution scheme for a silicic acid column to be used in conjunction with quantitative TLC follows that described by Rouser et al. (20) for the separation of phosphatidyl ethanolamine and phosphatidyl serine from beef brain. The silicic acid column is dehydrated and deoxygenated, packed in the chromatography tube to a height of 20 cm in chloroform/methanol 4/1, lipids applied in chloroform/methanol 4/1, and chloroform/methanol 4/1 used for the elution of two separate peaks, the first composed of a mixture of cholesterol, cerebroside, ceramide, phosphatidic acid, diphosphatidyl glycerol (cardiolipin), and sulfatides followed by another fraction containing phosphatidyl ethanolamine and phosphatidyl serine. The last fraction is eluted with methanol and contains lecithin, sphingomyelin, phosphatidyl inositol, the gangliosides, and water-soluble nonlipids. (With some silicic acid preparations sulfatides may be eluted with phosphatidyl ethanolamine and phosphatidyl serine.) Sample size and elution vols depend upon column diam. In this laboratory columns 2.5 (i.d.) x 20 cm with a flow rate of 3 ml/min are commonly used with from 100–300 mg of brain lipid applied. The fraction vols are col-

lected on the basis of a solids test (11) for each new lot of silicic acid since different preparations give different results. A cellulose column (see above) can be used prior to silicic acid column chromatography to remove gangliosides and water solubles. The silicic acid column can be prepared in chloroform, the sample applied in chloroform, and neutral lipids eluted with chloroform followed by from 10–20% methanol in chloroform for intermediate fractions, and methanol to clear the column. Various chloroform/methanol/water and/or one of the acidic solvent systems (see above) are used for quantitative TLC.

Magnesium Silicate-TLC Combinations

Neutral lipids can be eluted from dry magnesium silicate columns with chloroform (6 column vols), chloroform/methanol 95/5 (8 column vols) is then used to elute ceramide (and lipids of similar polarity), chloroform/methanol 2/1 (10 column vols) for the elution of cerebroside and sulfatide (also galactosylglycerides and sulfolipid), methanol (10 column vols) for elution of phosphatidyl ethanolamine and the gangliosides, and the column cleared with chloroform/methanol 2/1 saturated with water (11). Five percent (by vol) 2,2-dimethoxypropane is added to all solvents except chloroform/methanol/water. The elution vols and samples sizes depend upon the column diam. We utilize columns 2.5 (i.d.) x 10 cm with a flow rate of 3 ml/min and 100—200 mg of brain lipid as sample. The amounts of individual lipid classes in each column fraction is then determined by one-dimensional quantitative TLC. The methanol and chloroform/methanol/water eluates contain some adsorbent, but this adsorbent will not interfere with quantitative TLC if all the solids are dissolved before spotting onto plates or lipid loss onto discarded adsorbent is prevented. Quantitative TLC possesses the advantage that nonlipid materials that do not char do not interfere with the quantitative determinations.

A very simple elution scheme for brain lipids that may also be useful for other samples employs elution of neutral lipids with chloroform, followed by elution with methanol, and then chloroform/methanol 2/1 saturated with water. This gives three easily quantitated fractions.

Advantages of Column Chromatography Combined with Quantative TLC

The principal advantages are:

1) Overlapping spots on thin layer chromatograms are greatly reduced in number by preliminary column chromatography.

2) Minor components may be concentrated readily in a column fraction and then quantitated by TLC while with TLC alone they might go undetected or give such a small charring response that they could not be determined.

3) Identification of lipid classes can be made more certain with column-TLC combinations.

4) Simple column elution schemes can be utilized relative to those necessary for complete separation of lipid classes by column chromatography alone.

5) If column performance is imperfect and some fraction overlap is obtained, this can readily be compensated for by determining the amount of each lipid class in the area of overlap and adding to the appropriate major fraction.

6) With column-TLC combinations the presence of adsorbent or other nonlipid impurities (that do not char) in column fractions need not interfere with precise quantitative determinations.

General Summary of Available Chromatographic Methods

Three general procedures, column chromatography alone, TLC alone, and combinations of column and TLC are available for determination of polar lipids. The all column procedures are the means for careful standardization of all other procedures, for the isolation of adequate amts of material for characterization by acceptable methods, and for determination of fatty acid composition. The more rapid TLC methods are highly desirable for routine analytical use when carefully checked and the limitations of the procedures defined. The several combinations of column chromatography and TLC will perhaps prove to be the most generally useful because the precision of column chromatography and the speed of TLC are utilized. The rapid methods employing only TLC should be used with caution. Serious mistakes may be made and the occurrence of new substances, even in such frequently examined tissues as liver (3), will present major problems.

ACKNOWLEDGMENTS

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