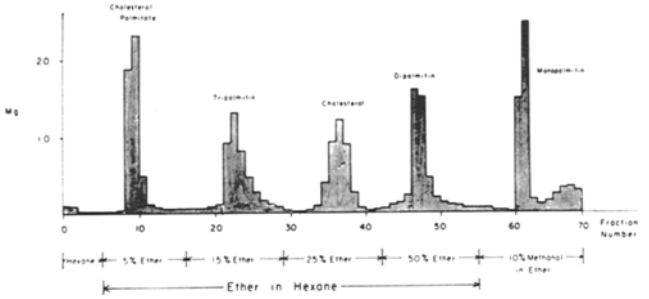


FIG. 9. Separation of lipid classes on a 30gm column of Florisil by stepwise elution. The column load consisted of 40mg of each component (10).

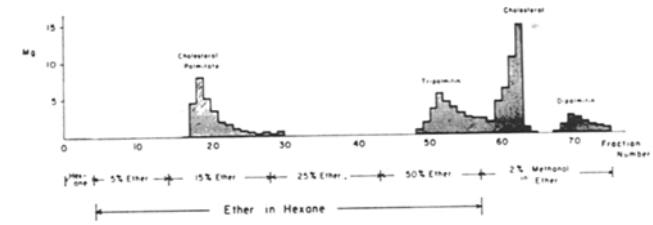


FIG. 10. Separation of lipid classes on a 12gm column of highly active Florisil. The column load consisted of 30mg each of cholesteryl palmitate, tripalmitin and cholesterol, and 15mg each of dipalmitin and monopalmitin (10).

so strongly that it was incompletely separated from cholesterol. When this new material was deactivated with 7% water, good separations were again observed (Fig. 11). Mixtures of naturally occurring lipids of rat blood and liver were well resolved and excellent recoveries were obtained. Studies indicate this method to be an adequate one for separation of human serum and artery lipids.

Florisil has some distinct advantages over silicic acid as an adsorbent for lipids. The rather large particle size (60–100 mesh) permits rapid flow rates without apparent loss of adsorptive surface and the material requires a minimum of preparation for use. An interesting property of this adsorbent is its affinity for free fatty acids. Elution of these lipids is delayed

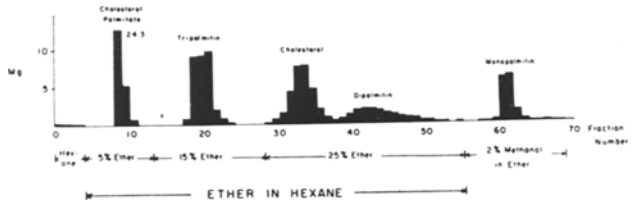


FIG. 11. Separation of lipid classes as in Fig. 10 after deactivation of the Florisil with 7% water (10).

until after monoglyceride, instead of emerging just before cholesterol as previously discussed with respect to silicic acid. This suggests the use of Florisil as a possible selective filter for free acids but presents a problem from the standpoint of recovery, as the addition of acetic acid to the solvent was required for their elution. Although Florisil may have some advantages over silicic acid, the problem of controlling the state of hydration is just as acute.

Whether one chooses Florisil or silicic acid it is obvious that these techniques require rigid control measures. But when the basic fundamentals of good column chromatography are observed, their use for separation of lipids can be a valuable addition to the technical knowledge of a laboratory.

REFERENCES

1. Winterstein, A., and Stein, G., Z. Physiol. Chem., *220*, 247 (1933).
2. Trappe, W., Z. Physiol. Chem., *273*, 177 (1942).
3. Brockmann, H., and Volpers, F., Ber., *80*, 77 (1947).
4. Hess, W.C., J. Lab. Clin. Med., *32*, 1163 (1947).
5. Reichstein, T. and Schoppee, C.W., Discussions Faraday Soc., *7*, 305 (1949).
6. Kellie, A.E., and Wade, A.P., Biochem. J., *53*, 582 (1953).
7. Kerr, L.M.H., and Bauld, W.S., Biochem. J., *55*, 872 (1953).
8. Trappe, W., Biochem. Z., *305*, 150 (1940).
9. Trappe, W., Biochem. Z., *306*, 316 (1940).
10. Carroll, K.K., J. Lipid Res., *2*, 135 (1961).
11. Börgstrom, B., Acta Physiol. Scand., *25*, 111 (1952).
12. Fillerup, D.L., and Mead, J.F., Proc. Soc. Exptl. Biol. Med., *83*, 574 (1953).
13. Luddy, F.E., Barford, R.A., Riemenschneider, R.W., and Evans, J.D., J. Biol. Chem., *232*, 843 (1958).
14. Hirsch, J., and Ahrens, E.H., Jr., J. Biol. Chem., *233*, 213 (1958).
15. Horning, M.G., Williams, E.A., and Horning, E.C., J. Lipid Res., *1*, 482 (1960).
16. Hanel, H. K., and Dam, H., Acta Chem. Scand., *9*, 677 (1955).
17. Van Handel, E., and Zilversmit, D.B., J. Lab. Clin. Med., *50*, 152 (1957).
18. Kay, L.M., and Trueblood, K.N., Anal. Chem., *26*, 1566 (1954).

Quantitative Chromatographic Fractionation of Complex Lipid Mixtures: Brain Lipids[1]

GEORGE ROUSER, A.J. BAUMAN, GENE KRITCHEVSKY, DOROTHY HELLER, and JOHN S. O'BRIEN,
Department of Biochemistry, City of Hope Medical Center, Duarte, California

THE OBJECTIVE of this presentation is to describe methods for the quantitative fractionation of brain lipids by means of column chromatography. It is not possible to give detailed directions for the chromatographic separation of complex lipid mixtures in general, as the types and quantities of lipids in mixtures from different sources may vary widely. Methods that are suitable for brain lipids may require modification for other lipid mixtures. A discussion of this complex subject must be approached in a general way with emphasis upon the variables that are involved in column chromatography, the means by which these variables can be controlled, and the mechanisms involved in the chromatographic processes. When these factors are appreciated, the extension of methods devised for brain lipids should be possible.

Emphasis has been placed on the use of infrared

[1] With comments on the mechanisms of chromatography and the use of infrared spectroscopy.

spectroscopy in column chromatographic studies. Infrared spectroscopy is an invaluable aid in characterization, and can be applied as a rapid means for determining the nature of substances eluted from columns. The general use of paper chromatography in the development of column chromatographic procedures, for monitoring of column fractions, and for the detection of impurities in lipid preparations will not be considered in detail as it has been reported on elsewhere (1,2,3). It should be noted however that paper chromatography can be instrumental in the development of column chromatographic procedures, as variables that are encountered in column chromatography may be explored with the more rapid paper methods.

General Techniques in Column Chromatography

The objective was the development of simple, rapid, and reproducible methods that can be carried out with readily available equipment. The glass chromatographic tubes are fitted with Teflon stopcocks. The stopcock should have a needle valve for adjustment of flow rate; it can then be used to start or stop the flow of solvent without change in flow rate. The narrow portion between the stopcock and the main body of the tube is generally made as small as possible in order to cut down the holdup volume in this dead space. The adsorbent is retained within the column by a thin layer of glass wool placed over a small glass cane fitted with an uneven bulb at the top (to avoid a tight fit with decrease of solvent flow) and inserted above the stopcock. The glass wool pad should be held in place from above with a glass rod until a small layer of adsorbent has been packed over it to prevent appearance of "fines" in the column effluent. This arrangement is preferred to sintered glass discs as the discs may become clogged with fine particles of adsorbent and the flow rate may be reduced to an impractical level. Flow rates are difficult to regulate with stopcocks when sintered glass discs are used because solvent and air back up behind the stopcock in an irregular manner.

A suitable flow rate may be obtained with some adsorbents without the use of pressure. When pressure is required it can be supplied by inserting a carefully prewashed black rubber stopper fitted with a glass tube into the top of the chromatography tube to transfer pressure from a prepurified nitrogen cylinder.

Automatic fraction collectors have not been used. It was preferred to collect individual fractions in glass stoppered, graduated tubes by hand. Each fraction is tested immediately after collection. With this method a solvent change can be made as soon as elution of one fraction is complete without collection of unnecessary fractions. Once the elution scheme is worked out, fractions may be collected in bulk and the amount of material can be determined by weight.

A solids test is a simple, rapid, general method for monitoring column fractions. A test for solids in the chromatographic effluent can be carried out very conveniently with 1 ml. of a column fraction. The aliquot is evaporated to dryness in a few seconds in a test tube inserted in a very hot (200–300°C.) sand bath. The tube is cooled and held up to the light for the examination of residual solids. With practice this simple technique can be used to detect any lipid or other substance emerging from the column in very small amounts. We have found that we can detect material by eye in fractions that contain only an insignificant weight of total solids. Inorganic material present in the sample may be eluted along with lipid, or part of the column material may appear in fractions. Inorganic substances can be differentiated from lipid by allowing the tube to remain in the hot sand bath for several minutes. Organic material will char. Such a simple, rapid test can be carried out on each fraction from a column prior to the complete emergence of the next fraction. This makes a rapid change of solvent possible and facilitates the development of the most rapid and efficient elution scheme. The solids may be used for infrared examination and a rapid scan can give valuable information about column performance during a run.

The solids test may readily be made quantitative by weighing. A solids curve (plot of mg. of lipid *versus* volume of effluent) is an excellent means of illustrating column performance. The procedure is carried out by weighing a small aluminum cup (about 10 mg., preheated and dried in a desiccator) with a Cahn microbalance, evaporation of 1 ml. of column effluent in the cup in a warm sand bath, cooling in a desiccator, and reweighing the pan. The entire procedure requires about 5 min. per sample, and reliable weights are obtained. Individual fraction weights determined in this manner, and added to give a total amount of lipid in a peak, agree within 1 to 2% of the weight determined by pooling all fractions, evaporation of solvent, and weighing on an ordinary analytical balance.

It may be necessary to conduct all of the operations under pure nitrogen. Solvents can be deoxygenated under reduced pressure from a water pump, followed by return to atmospheric pressure with pure nitrogen. Solvents can be kept air-free by passing a slow stream of nitrogen bubbles through the solvent prior to pouring into the chromatography tube. Column packing such as silicic acid may be deoxygenated by placing the adsorbent in a three-necked flask fitted with a thermometer, an inlet tube for pure nitrogen, and a tube for attachment to a water pump. The flask is heated with a heating mantle, and rapid deoxygenation is insured by reduced pressure while a slow stream of nitrogen is passed over the surface of the adsorbent. After several hours the vessel is returned to atmospheric pressure with pure nitrogen and allowed to cool before opening. When cool, the adsorbent is transferred to a suitable storage flask as a slurry in deoxygenated solvent. Such slurries can remain essentially oxygen free in tightly fitting glass stoppered bottles for weeks.

Many laboratory operations should be carried out under nitrogen. Since working in a nitrogen filled box through gloves is awkward and time consuming, other simple, rapid methods can be substituted. Filtration can be accomplished without exposure of the supernatant solution or residue to air by inverting a funnel equipped with a rubber stopper and a nitrogen inlet tube over the filter. The funnel is placed close to the filter and acts as a cover and nitrogen is supplied to keep air out. The investigator can easily add more solution, etc. Transfers can be made under nitrogen by placing the equipment beneath a mantle prepared from a plastic bag open at one end and fitted to a nitrogen cylinder on the other end. A slight positive pressure may be maintained and, for

the most careful work, manipulations can be carried out through the plastic bag. Fractions may be collected from a column in a similar manner by fitting the end of the column with a suitable mantle that is filled with nitrogen. A slow flow of nitrogen through the system is usually maintained. Similarly, glass-stoppered test tubes placed in a test tube rack may be stored conveniently under nitrogen by placing the rack in a plastic bag filled with nitrogen. Lipid preparations that must be sampled frequently should be stored in a carefully greased, smooth fitting vacuum desiccator. The desiccator should be pumped down with a vacuum pump, filled with nitrogen, evacuated again, and refilled with nitrogen. Lipid samples stored in glass ampoules sealed under nitrogen can be kept indefinitely and require a minimum of space. Ampoule storage is too time consuming for preparations to be used each day where desiccator storage is practical.

Analytical columns can be run conveniently in chromatography tubes of 2.5 cm. i.d. Two convenient lengths are 20 and 40 cm. Used here was a 40 cm. tube packed to a height of 20 cm. with diethylaminoethyl (DEAE) cellulose (15 g.) and silicic acid (55–60 g.) and 20-cm. tubes filled to a height of 10 cm. for magnesium silicate and silicic acid-silicate-water columns. No more than 350–400 mg. of crude brain lipid is applied to DEAE columns of this size. The loading factor for the other columns varies and depends upon the particular mixture to be fractionated. Flow rates of about 3 ml./min. are maintained. With the 2.5-cm. columns, 10-ml. fractions are suitable and bulk fractions can be collected after column performance has been standardized.

Preparation of Samples for Infrared Examination

Spectra of lipids in various solutions or from Nujol mulls are useful, but a complete spectral scan from 2–15 μ is best obtained from samples pressed into micropellets with KBr or spread as thin films over silver chloride plates. Micropellets are prepared from 10 mg. of KBr and from 50–300 μg. of lipid. The weighing is done with a Cahn (or similar) microbalance. The sample is ground into the KBr in an agate mortar and transferred to a Beckman micropellet die. After the sample is pressed in the hydraulic press, the thickness of the pellet is measured with a micrometer, and the spectrum is recorded on a Beckman IR-4 infrared spectrophotometer.

Some lipids are difficult to prepare in the pellet form. In cases of this sort, as well as with extremely small samples and as a check on the KBr technique, a silver chloride plate is used. The sample is spread as a film either by mechanical means or by transfer with a suitable solvent.

Extraction of Beef Brain Lipids

Quantitative determinations must be based on quantitative lipid extraction procedures that are performed in such a way that oxidation, hydrolysis, etc. are avoided. A satisfactory procedure follows. The fresh whole brain is homogenized with solvent immediately after removal from the animal or cut into small pieces, placed in a plastic bag, and frozen over solid CO_2 for transport to the laboratory and then homogenized. Homogenization is carried out in a Waring blendor using 20 ml. of deoxygenated chloroform/methanol, 2/1, (v/v) for each gram wet weight of tissue. The space above the solvent is filled with nitrogen and the top of the homogenizer, if not made of glass, is protected by covering with aluminum foil and clamped down tightly. The mixture is homogenized for 3–5 min. at room temperature and then transferred to a sintered glass filter. Filtration is accomplished under nitrogen as described above. The residue is re-extracted twice with half the original volume of solvent. Evaporation of the extracts under nitrogen at 0–15°C. is then accomplished in a rotary evaporator flushed periodically with nitrogen. The time required is reduced if the final extract is evaporated first, then the second, and finally the original extract. The solids are dried by placing the flask and contents in a vacuum desiccator over KOH. The pressure is reduced with a vacuum pump and the desiccator is left at room temperature for 10–18 hrs. The dry lipid is then re-extracted 3 times with one-liter portions of chloroform/methanol, 2/1, with filtration as above at each step. This step removes an insoluble residue of protein. The lipid is recovered by evaporation of solvent under nitrogen as described above except that the mixture is not taken to dryness. When the mixture has reached a small volume with a good deal of suspended lipid, it is transferred (under nitrogen) to a large mortar. The mortar is then placed in a vacuum desiccator equipped with a nitrogen inlet capillary and attached to a water pump. Evaporation and drying are carried out under a slow stream of nitrogen and reduced pressure. The thoroughly dry lipid is then mixed in the mortar with a pestle, scraped with a spatula, reground with the pestle, etc. until the sample is homogeneous. The latter operations can be performed under nitrogen by working under a plastic bag mantle filled with nitrogen as described above. The lipid is then divided into the desired portions and sealed in pyrex glass ampoules under nitrogen for storage.

This method of extraction appears to be quantitative as far as the lipids listed in Table I are concerned. The extract does not appear to contain the complex di- or triphosphoinositides.

General Approach to Quantitative Fractionation

The approach to the development of procedures for the fractionation of complex lipid mixtures involves a preliminary subfractionation of the complex mixture into smaller groups of lipids by stepwise elution, followed by separation of each of the subfractions into individual lipid classes on other column materials most suitable for the particular mixture. Most investigators have used another approach. One column material only is used and elution is begun with nonpolar solvents such as hexane and then with solvents and solvent mixtures of increasing polarity ending with an alcohol to strip the column. No single column substance is known from which all of the lipid classes can be eluted in pure form. For this reason previous attempts to separate complex mixtures have not been completely successful and many times investigators have not been aware of the fact that mixtures were obtained due to inadequate means for evaluating purity. Gradient elution has been used a great deal. This means of elution is useful for rapid preliminary determinations of the properties of a particular column packing and for rapid elution of substances of widely different properties, but it is less useful than stepwise elution in most instances when complete separation

with quantitative recovery is desired, as peaks are pushed into each other by gradient elution.

When the multicolumn procedure is used, it is possible to establish optimum conditions for the elution of each lipid class. The lipid classes that are not separable on one column are separated on another type of column. It is sometimes possible to obtain complete separation of two substances by using a relatively poor eluting solvent that will bring both substances off separately as long, low peaks. This is a rather unprofitable approach as it is time consuming, and column material and solid residue from solvents may seriously contaminate the fractions. We prefer to elute such substances together for fractionation on another type of column where the difference in elution properties is greater.

The steps in the determination of the simplest possible means by which a complex mixture can be fractionated are as follows. First, a variety of column packings are studied. Studies were begun with silicic acid, the most commonly used substance. After an analysis of the factors involved in silicic acid column chromatography was completed, a new type of column was devised. It was called a silicic acid-silicate-water column from the fact that a silicic acid column was prepared containing some silicate and water, both added in carefully controlled amounts. This new type of column made possible several separations that were not obtainable on silicic acid. Also, various ion exchange celluloses were explored and useful methods for lipids in nonaqueous media were successfully developed. These studies included work with carboxymethyl cellulose, diethylaminoethyl (DEAE) cellulose, triethylaminoethyl cellulose, phosphocellulose, and sulfoethyl cellulose. From these studies we determined that DEAE cellulose was one of the most useful materials. Further investigation was made of the use of magnesium silicate, a very strong adsorbent, to determine the manner in which all of the brain lipids could be eluted from wet and dry columns.

From these studies it was possible to put together an elution scheme for the essentially complete fractionation of brain lipids starting with DEAE cellulose for preliminary subfractionation of the mixture. Pure phosphatidyl ethanolamine is eluted from DEAE and the water-soluble, nonlipid contaminants (free of lipid) are obtained in a single fraction. The other fractions from the DEAE column are then separated into individual lipid classes using either silicic acid-silicate-water columns or silicic acid columns. These procedures are described in more detail below. Other column packings including magnesium silicate and various ion exchange celluloses are of value as independent check methods. The values for cholesterol, ceramide, cerebroside, and cerebroside sulfate can be checked with magnesium silicate columns; values for phosphatidyl ethanolamine and phosphatidyl serine by a combination of silicic acid and silicic acid-silicate-water column chromatography as previously described (2); and gangliosides can be isolated from cellulose columns.

Results with Beef Brain

The average values obtained for beef brain lipids from many column runs are shown in Table I. It was not known at the beginning of the work how many unknown substances might be encountered as previous studies were of a nonquantitative nature. Despite the fact that brain has been a very common source for the isolation of lipids by solvent precipitation procedures, column chromatography has not been applied to any great extent. No reports have been found in the literature from other laboratories that describe any serious attempt to separate the brain lipid mixture into individual lipid classes with quantitative recovery.

TABLE I

Composition of Whole Beef Brain Lipid

	Substance	% Total lipid
(1)	Cholesterol	20.3
(2)	Ceramide	0.3
(3)	Cerebroside	16.8
(4)	Cerebroside sulfate	3.5
(5)	Lecithin	11.0
(6)	Lysolecithin	Trace
(7)	Sphingomyelin	7.9
(8)	Phosphatidyl ethanolamine	17.0
(9)	Phosphatidyl serine	7.2
(10)	Inositol phosphatide	3.1
(11)	Uncharacterized (3 components)	2.8
(12)	Ganglioside	2.8
(13)	Water soluble nonlipid	7.0
		99.7%
	Sphingolipid (2,3,4,7,12)	31.3%
	Glycerophospholipid (5,8,9)	35.2
	Incompletely characterized (10,11)	5.9
	Water soluble (13)	7.0
	Sterol (1)	20.3
		99.7%

Table I does not show substances that probably occur in extremely small amounts (0.1% or less) in brain lipid. Small amounts of saturated and unsaturated hydrocarbons have been reported to be present in beef brain (4) and a small amount of coenzyme Q has been determined by a very delicate procedure (5). It is possible that other substances may be present in some of the column chromatographic fractions in very small amounts. Hydrocarbons and coenzyme Q should appear in the cholesterol fraction in our present elution scheme. It is estimated that the cholesterol value is not off by more than 0.2 (in terms of total amount of brain lipid) as a result of such contamination. Careful paper chromatographic and infrared examinations indicate that the cholesterol fraction probably does not contain more than 2% of its weight of impurities.

There is a significant percentage of partly or completely uncharacterized material. The inositol phosphatide recovered from brain lipid mixtures prepared by exhaustive extraction with chloroform/methanol, 2/1, is not the complex inositol phosphatide described by Folch (6) or the triphosphoinositide described by more recent investigators (7). The inositol phosphatide isolated from DEAE (fraction 6, Table II) has the chromatographic characteristics of phosphatidyl inositol (isolated from soybean) and is tentatively identified as phosphatidyl inositol.

A fraction eluted from DEAE with chloroform/acetic acid is incompletely characterized, but contains at least three separate substances. This fraction is quite labile. One of the constituents has chromatographic properties of an oxidation product of phosphatidyl ethanolamine and is ninhydrin positive. A small amount of free fatty acid is probably present in this fraction, but the major component is completely uncharacterized.

The values shown in Table I for cholesterol, ceramide, cerebroside, cerebroside sulfate, phosphatidyl ethanolamine, phosphatidyl serine, ganglioside, and water soluble nonlipid contaminants are believed to

be quite accurate. The values for lecithin and sphingomyelin may be in somewhat greater error (due to the small number of determinations) and the value for the inositol phosphatide has not been established as entirely accurate. The individual percentages of the uncharacterized substances have not been determined with accuracy due to their considerable lability.

Silicic Acid Column Chromatography

These studies were begun with silicic acid column chromatography. The objectives were control of the variables and evaluation of the usefulness of this substance for the separation of lipids from complex mixtures. A number of valuable observations had been recorded in the literature. Borgström (8) reported that neutral lipids could be separated from phospholipids on silicic acid by elution of neutral lipids with chloroform, followed by elution of phospholipids with methanol. When this procedure is applied to brain lipids, several nonphospholipids are eluted along with the phospholipids. These include cerebrosides, sulfatides, and gangliosides. Other investigations extended the knowledge of the manner in which lipids may be eluted from silicic acid. One successful elution scheme for the neutral lipids (nonphospholipids) was that of Barron and Hanahan (9). As with previous methods, the procedure was useful for the elution of hydrocarbons, sterol esters, triglycerides, fatty acids, sterols, and mono- and diglycerides by elution successively with hexane, benzene/hexane mixtures, benzene, diethyl ether/hexane mixtures, and diethyl ether.

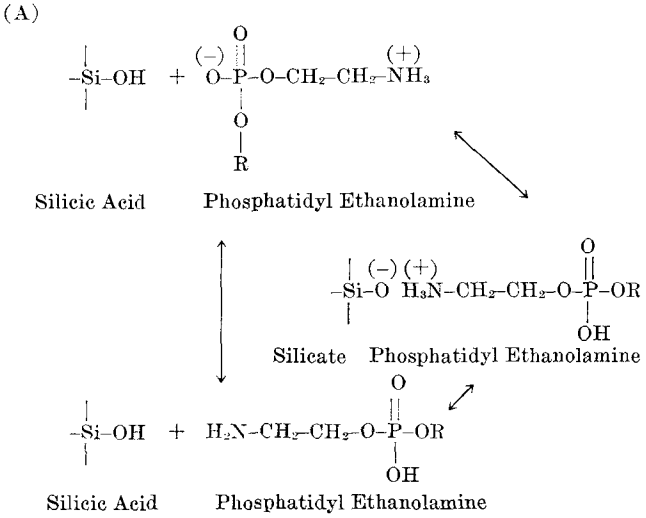
The more difficult problem has been the separation of individual phospholipids on silicic acid columns. Lea *et al.* (10) were the first to report a useful procedure for the separation of individual phospholipid classes on silicic acid columns. These investigators were able to isolate pure phosphatidyl ethanolamine from egg yolk lipid. The method of Lea (11) was an improvement over the original procedure in that autoxidation of phosphatidyl ethanolamine was avoided by exclusion of oxygen during the chromatographic separation. When these methods are applied to brain lipids, pure phosphatidyl ethanolamine is not obtained, as phosphatidyl serine (not present in egg yolk lipid) is eluted in the same fraction. Hanahan *et al.* (12) extended the elution scheme for silicic acid columns and were able to elute a pure phosphatidyl inositol from liver lipid. Other investigators have reported the use of silicic acid columns for the fractionation of lipid mixtures from various sources, but surprisingly, as noted above, no serious attempts to fractionate brain lipids by column chromatography have been reported other than those from this laboratory (2,3,13).

All the early studies were complicated by a lack of reproducibility and incomplete separation of the components of complex mixtures. Lack of reproducibility is produced by failure to appreciate and/or control the variables in a procedure. The variations in silicic acid column chromatography have been traced to: (1) water content of the system; (2) silicate content of the silicic acid; (3) particle size variations of the adsorbent; (4) amount of free salt (largely NaCl) in silicic acid; (5) the loading factor (amount of lipid applied). It is necessary to describe procedures in detail to control these variables.

The adsorption properties of silicic acid appear to decrease as water content is increased. Most silicic acid however is not pure but contains a considerable quantity of silicate. Silicate binds water firmly, and we have discovered that lipids may be bound through water to the silicate (3,14). Retention of lipids by the stationary phase may thus be increased by water. It is possible to flood a silicic acid-silicate system with so much water that the capacity is decreased.

An appreciation of the role of particle size of the adsorbent, and of what is meant by overloading, can be obtained only when knowledge of the forces involved in silicic acid column chromatography is available. Silicic acid columns appear to function largely by ion exchange and hydrogen bonding. The ion exchange reactions of silicic acid can be illustrated by the behavior of phosphatidyl ethanolamine on silicic acid columns. Phosphatidyl ethanolamine in a chloroform-methanol mixture can undergo ion exchange reactions with silicic acid, and the proton from the silicic acid is transferred to the ionized phosphate group of phosphatidyl ethanolamine in the reaction. The anionic site thus created on the adsorbent is then balanced by the $-NH_3^{(+)}$ grouping of phosphatidyl ethanolamine. As the positively charged amino group may then donate a proton back to the silicate ion produced in the original ion exchange reaction, its binding tendency is decreased. These reactions are illustrated in (A) below.

(A)



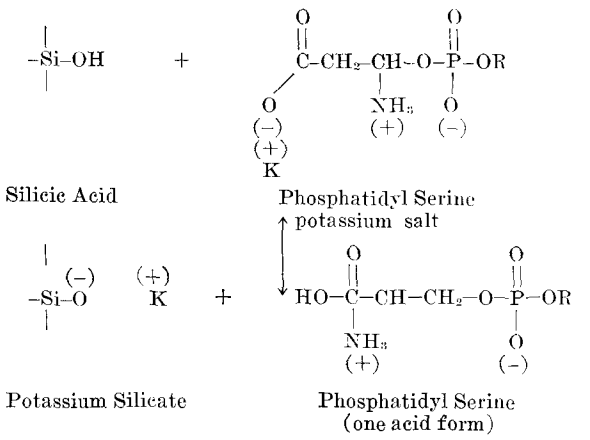
A similar ion exchange reaction with lecithin results in a stronger bonding of this phospholipid to the negatively charged site on the adsorbent because the choline quaternary ammonium nitrogen cannot donate a proton. The lecithin molecule can become detached from the negative binding site if the proton of the phosphate group is returned to the silicate ion. This offers less possibility for escape than for phosphatidyl ethanolamine and lecithin is retained more firmly by the adsorbent.

The role of hydrogen bonding is illustrated by the difference in the elution characteristics of a number of compounds. Lysolecithin is eluted from silicic acid columns after lecithin. The ion exchange reactions are presumably the same for both molecules, but the presence of an hydroxyl group in lysolecithin creates an additional possibility for hydrogen bonding to silicic acid. Cholesterol (one hydroxyl group) may be bound by hydrogen bonds to silicic acid and can be eluted readily with mixtures of ether and hexane. A substance such as lysolecithin that inter-

acts by ion exchange and hydrogen bonding requires a chloroform/methanol mixture with a large amount of methanol for elution. The total number of groups, and hence the total hydrogen bonding capacity, determines the ease of elution from silicic acid. Cholesterol with one hydroxyl group is rapidly eluted with ether, while cerebrosides containing several hydroxyl groups are not. Cerebrosides are eluted with solvent mixtures containing alcohols. Phosphatidyl inositol and cerebroside have the same total number of free hydroxyl groups but the phosphate group of the phospholipid may hydrogen bond, and this probably accounts, at least in part, for the elution of phosphatidyl inositol well behind cerebroside. Phosphatidyl inositol is eluted rapidly only with a much higher concentration of methanol in chloroform.

The major defect of silicic acid columns for the separation of complex lipid mixtures is the overlap of acidic lipids with nonacidic lipid fractions. One of the reasons for this is illustrated by the behavior of phosphatidyl serine. Phosphatidyl serine is usually obtained by extraction with neutral solvents and is probably present largely as the potassium salt. The salt of phosphatidyl serine undergoes an ion exchange reaction with silicic acid in which there is a proton transfer from silicic acid to the carboxyl group of phosphatidyl serine. The potassium is then bound to silicate as illustrated in diagram (B).

(B)

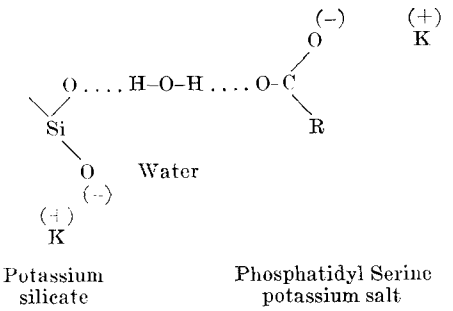


The undissociated carboxyl group does not influence the elution of phosphatidyl serine from silicic acid columns to any great extent and the substance is eluted along with phosphatidyl ethanolamine. There may be either partial or complete overlap with phosphatidyl ethanolamine depending upon the exact conditions. On a pure silicic acid column (free of silicate) that may be produced by acid washing of the silicic acid in a chromatography tube followed by careful washing with deionized water, phosphatidyl serine tends to precede phosphatidyl ethanolamine. This behavior can be explained by assuming that the carboxyl groups of two phosphatidyl serine molecules engage in hydrogen bonding that interferes with the interaction of the molecules with the silicic acid.

Conditions influencing the load that can be applied to silicic acid columns can now be appreciated. A convenient amount of lipid can be applied to a silicic acid column when a moderately fine particle size is used. There must be enough surface for lipid binding. Silicic acid is relatively easy to fragment by mechanical means (as by forcing through a wire sieve or vigorous stirring) and very fine particles may be produced that will clog a column to produce slow flow rates (2). When the particle size is too large, fraction overlap results unless the amount of lipid applied is greatly reduced.

Overloading of a column may alter the chromatographic results to a marked extent. This is illustrated by the behavior of phosphatidyl serine when applied in large amounts to silicic acid columns. If a large amount of the potassium salt of phosphatidyl serine is applied, the ion exchange capacity of the silicic acid may be exceeded and the salt form of phosphatidyl serine will not be converted completely to the free acid form. Thus, two different ionic states of the molecule are produced and the silicate content of the column is increased by overloading. The two different forms of phosphatidyl serine can be separated on the columns. We have demonstrated that silicate in the presence of water may bind anionic groups of lipids very tightly. It is apparent therefore why early investigators such as Phillips (15) and Marinetti *et al.* (16) obtained more than one peak for phosphatidyl serine when the system was not anhydrous and the columns were overloaded. A portion of the phosphatidyl serine under such conditions is eluted in the usual position with chloroform/methanol along with phosphatidyl ethanolamine. A remaining strongly adsorbed portion of phosphatidyl serine is eluted with methanol or methanol/chloroform mixture containing large amounts of methanol. This latter fraction contains the potassium salt of phosphatidyl serine that is probably bound, at least for the most part, as shown:

(C)



A method has been described for the preparation of silicic acid with which oxygen, water, and free salt are removed to give very reproducible column performance (2). With all of these precautions however silicic acid is still of limited use for the general fractionation of complex lipid mixtures. A major defect, as pointed out above, is that acidic and nonacidic lipid fractions overlap. Cardiolipin, phosphatidyl serine, inositol phosphatides, sulfatides, and gangliosides are found to overlap with other lipids on silicic acid columns. The difference in the binding of sphingomyelin as compared to lecithin is not great enough for complete separation of these two substances. From these studies it became apparent that one of the major problems in the fractionation of complex mixtures is the complete separation of acidic lipids from nonacidic lipids. We studied several methods for this separation and were most successful with diethylaminoethyl (DEAE) cellulose as described below. The overall fractionation procedure for brain lipids does include several separations that are conveniently made on silicic acid. These separations are performed after a preliminary separation of lipids into suitable fractions with DEAE cellulose.

Diethylaminoethyl (DEAE) Cellulose Column Chromatography

An anion exchanger such as DEAE cellulose binds anionic substances tightly and this property has been used frequently with aqueous systems. It has been shown that anionic lipids may be bound to DEAE cellulose in nonaqueous systems. Ion exchange celluloses have been used with considerable success for the fractionation of large molecules such as proteins and nucleic acids. The ion exchange celluloses appear to be more useful than ion exchange resins for high molecular weight substances. The open nature of the cellulose and the greater availability of the ion exchange sites permits large molecules to come in contact with more of the available sites while eluting solvents can penetrate and break the bonds between adsorbent and the substance being chromatographed.

We have examined several ion exchange celluloses (3,14). Phospholipids are not retained appreciably by carboxymethyl cellulose or by dry sodium phosphocellulose. On the other hand, the salt form of phosphocellulose does retain lipids in the presence of water (3). The anion exchange celluloses including DEAE, Ecteola, and triethylaminoethyl cellulose (TEAE) can be used for the column chromatography of phospholipids. These exchangers do not require the presence of water for the retention of lipids and reproducible results may be obtained under carefully defined conditions. The DEAE cellulose has been selected as the most promising general anion exchanger. Ecteola appears to behave essentially like DEAE, and TEAE is similar to DEAE in many respects. An interesting difference between DEAE and TEAE cellulose is the fact that lipid is not bound to the chloride or acetate forms of TEAE, while the hydroxyl form of this ion exchanger readily binds lipid. Another important difference between the two ion exchange celluloses is that, while DEAE in the acetate form readily binds phosphatidyl ethanolamine in the absence of water, the hydroxyl form of TEAE binds phosphatidyl ethanolamine only in the presence of water. These differences offer additional possibilities for fractionation in special cases.

The acetate form of DEAE was chosen in order that no strong acid would be released during the ion exchange reactions. Strong acid can degrade labile forms of lipids (such as the plasmalogen forms of phosphatidyl ethanolamine). After a thorough prewashing with 1 *N* HCl followed by water and 1 *N* KOH, the DEAE cellulose is put in the acetate form by treatment with glacial acetic acid. The acetic acid is washed out with methanol and the column is prepared in a suitable chloroform-methanol mixture (usually chloroform/methanol, 7/1, v/v) before application of the sample.

The broadest elution scheme developed so far is shown in Table II. Pure phosphatidyl ethanolamine is obtained from DEAE and the water soluble, nonlipid contaminants are eluuted in a single fraction free of lipid. The four other fractions obtained however must be separated into the individual lipid classes by other means.

The zwitterion lipids (lecithin and sphingomyelin) and the nonionic lipids appear at the solvent front when the brain lipid mixture is applied to DEAE cellulose in chloroform/methanol, 7/1. All of the acidic components are firmly retained by the anion exchanger as expected. Fractions 5 and 6 and presumably fraction 4 of Table II are composed of acidic lipids. The fractions firmly bound to DEAE are elutable with acidic or basic solvents. The weakly acidic chloroform/acetic acid mixture elutes three uncharacterized components (free fatty acids when present are eluted with this solvent), and glacial acetic acid elutes two substances that are acidic by virtue of the carboxyl groups, namely phosphatidyl serine and ganglioside. A chloroform/methanol mixture containing aqueous ammonia is required for the elution of the strongly acidic cerebroside sulfate and inositol phosphatide (cardiolipin is eluted in this fraction when present).

TABLE II

Elution of Lipids from DEAE Cellulose (Acetate Form)

	Substance	Solvent
(1)	Lecithin, sphingomyelin, ceramide, cerebroside, cholesterol, lysolecithin, (also sterol ester and glycerides when present)	Chloroform/methanol, 7/1
(2)	Phosphatidyl ethanolamine	Chloroform/methanol, 7/3
(3)	Water soluble nonlipids (sugars, amino acids, purines, salt)	Methanol
(4)	Uncharacterized (3 components) also free fatty acids when present	Chloroform/glacial acetic acid (3/1) containing 0.001 M potassium acetate
(5)	Phosphatidyl serine and ganglioside	Glacial acetic acid
(6)	Cerebroside sulfate, inositol phosphatide (and cardiolipin)	Chloroform/methanol, 4/1, containing 10 ml./liter conc. aqueous ammonia

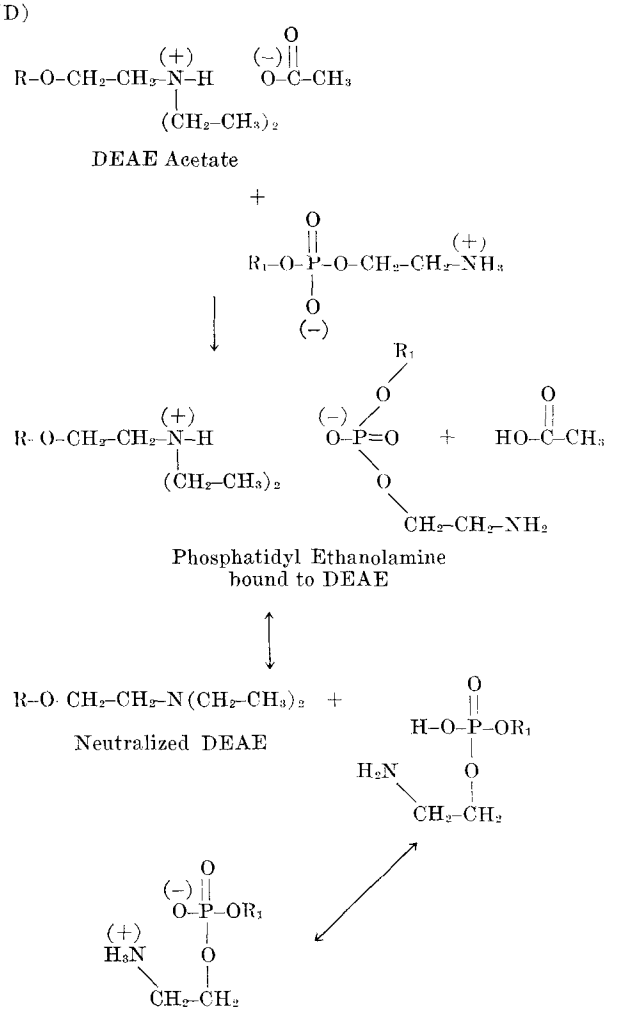
Simple ion exchange considerations would lead to the expectation that the zwitterion lipids, lecithin and sphingomyelin, that have both a positive and a negative charge should be retained only slightly by DEAE and that these lipids should be elutable with nonionic solvents such as mixtures of chloroform and methanol. In the ion exchange process the acetate ion must balance the quaternary ammonium grouping of choline and, as the process is readily reversible, the binding is weak. The nonionic lipids (cholesterol and cerebroside) pass through the ion exchange cellulose, as hydrogen bonding through hydroxyl groups is not strong enough in the presence of polar solvents (such as methanol) to cause any but the slightest retention. The possibility was explored of the differential elution of these nonionic lipids on DEAE cellulose with less polar solvents, but it was found that the separations could be obtained more conveniently by other means.

Phosphatidyl ethanolamine undergoes reaction with DEAE that results in stronger binding. A phosphate group can become attached to the adsorbent with exchange of the acetate ion for the phosphate ion. Acetic acid can be produced in the reaction as a result of the proton donor ability of the $-NH_3^+$ group of phosphatidyl ethanolamine. That phosphatidyl ethanolamine should be elutable with a nonionic solvent follows from the fact that DEAE is itself a proton donor. Escape from the positively charged site on the adsorbent is possible as the proton of the diethylaminoethyl group can be transferred to the phosphate group of phosphatidyl ethanolamine with the subsequent release of the phosphatidyl ethanolamine molecule from the adsorbent. A proton transfer from phosphate to $-NH_2$ to give $-NH_3^+$ would then restore the phosphatidyl ethanolamine to its original zwitterion form. These reactions are illustrated below (D). Evidence in favor of this simple interpretation has been obtained with TEAE cellulose. TEAE is not a proton donor and retains adsorbed phosphatidyl ethanolamine when treated with nonionic solvents.

TEAE cannot donate a proton to the phosphate group and phosphatidyl ethanolamine cannot escape unless an acidic or basic solvent is used for elution.

Acidic lipids in the salt form ion exchange with DEAE. The phosphate or other acidic groups interact with the positively charged site of the adsorbent with the production of, for example, potassium acetate which is washed through the column. Acidic lipids are retained very firmly and must be eluted from DEAE with acidic or basic solvents. When DEAE columns are overloaded, acidic lipids are not bound completely and may be eluted with nonionic solvents.

(D)



Silicic Acid-Silicate-Water Column Chromatography

A method has been described in detail for the complete separation and quantitative recovery of phosphatidyl ethanolamine and phosphatidyl serine using silicic acid-silicate-water columns (2). With this procedure phosphatidyl ethanolamine and phosphatidyl serine are first isolated together (free of other lipids) by silicic acid column chromatography. Phosphatidyl ethanolamine and phosphatidyl serine are then separated from each other with the new silicic acid-silicate-water column. The silicic acid-silicate-water columns for this separation are conveniently prepared by passing a mixture of chloroform/methanol/aqueous ammonia through a bed of silicic acid in a chromatography tube. This procedure for introducing a known amount of silicate and water is advantageous in that it can be carried out readily under oxygen-free conditions. The preparation of the column in this manner however produces a bed that contains a great deal more silicate and water at the top, and the amount drops steadily from the top to the bottom.

The silicic acid-silicate-water columns were devised after the analysis of factors involved in silicic acid column chromatography had been completed. The results with silicic acid columns were in keeping with other chromatographic data obtained in this laboratory with inorganic phosphates (sodium and potassium phosphates) and salts of phosphocellulose. With these chromatographic systems it appeared that anionic sites on the adsorbent, in the presence of water, bound polar and acidic lipids very firmly. Phosphatidyl ethanolamine is eluted with 20% methanol in chloroform in the same position from silicic acid-silicate-water and silicic acid columns. Phosphatidyl serine is more firmly bound to the silicate-water columns and is eluted with methanol rather than 20% methanol in chloroform that can be used to elute it from silicic acid.

Phosphatidyl ethanolamine and phosphatidyl serine are relatively labile lipids and undergo relatively rapid autoxidation and the chromatographic separation must be carried out in a nitrogen atmosphere. The method of Lea (11) that uses the absorption at 235 and 275 mμ due to conjugated double bonds as a measure of autoxidation is of great value in the study of these two lipids. Low molecular extinction coefficients may be obtained only when the silicic acid and silicic acid-silicate-water columns are free of oxygen and pure nitrogen is used in all of the operations.

A more recent development in this laboratory has been the extension of the use of silicic acid-silicate-water columns for the separation of the front fraction from DEAE into its individual lipid components (cholesterol, ceramide, cerebroside, lecithin, and sphingomyelin) as shown in Table III. Although choles-

TABLE III

Elution from Silicic Acid-Silicate-Water Columns

Solvent	Substance
Chloroform	Cholesterol
Chloroform/methanol, 19/1	Ceramide
Chloroform/methanol, 4/1, + 0.5% water	Cerebrosides
Chloroform/methanol, 4/1, + 1.5% water	Lecithin
Chloroform/methanol, 4/1, + 2.0% water	Sphingomyelin
Methanol + 2% water	Lysolecithin

terol, ceramide, and cerebroside can be separated with magnesium silicate columns, silicic acid-silicate-water columns are of particular advantage as lecithin and sphingomyelin are completely separated on them in addition to cholesterol, ceramide, and cerebroside.

The silicic acid-silicate-water columns in this case are prepared in a different way from those used for the separation of phosphatidyl ethanolamine and phosphatidyl serine. A column with greater capacity is required. The increased capacity is conveniently produced by treating a slurry of acid washed silicic acid in chloroform/methanol, 1/1, with concentrated aqueous ammonia (for each 50 g. of silicic acid 10 ml. of concentrated aqueous ammonia was used). The mixture of silicic acid and ammonium silicate is then transferred to a chromatography tube, and water and methanol are washed out by passing chloroform through the columns. The lipid sample is then ap-

plied in chloroform, and elution is carried out as indicated in Table III. It is emphasiized that successful elution requires the addition of water in carefully controlled amounts to the chloroform/methanol mixture.

The silicic acid-silicate-water columns appear to function in part as partition chromatography columns. This means that there is a stationary liquid phase and that the substance being chromatographed is bound to the adsorbent through solvent molecules (partition chromatography) rather than directly to the adsorbent as in adsorption chromatography. In this case, lecithin and sphingomyelin are bound to silicate through water molecules. A pure silicate column with a monovalent ion (such as the ammonium ion) is of limited value for the separation of lecithin and sphingomyelin as such columns have a very limited capacity. A mixture of silicic acid and silicate is advantageous because the ion exchange properties of the silicic acid are retained and the system has a high capacity, while the hydrogen bonding properties of the hydroxyl group of sphingomyelin are brought out by virtue of silicate-water-hydroxyl group interaction. Lipids are bound less firmly to the stationary partition phase and the peaks do not have the long tailing portions characteristic of pure adsorption columns. The tailing portion in adsorption chromatography is related to the fact that at low concentration more adsorption sites are available and fewer molecules of the substance being chromatographed remain in the mobile phase (the ratio of the amount of substance adsorbed per gram of adsorbent to the concentration in solution increases as the concentration in solution is decreased).

Magnesium Silicate Column Chromatography

Radin *et al.* (17) and Kishimoto and Radin (18) have described procedures using Florisil (a commercial preparation of magnesium silicate) for the separation of cerebrosides from brain lipid. In efforts here the Radin procedures did not yield pure substances. A good deal of the column material was eluted along with lipid and, as peaks had long leading portions, fraction overlap was obtained. It was possible to overcome these objections and determine the conditions necessary for the elution of the various lipids of beef brain as shown in Table IV. The appearance of column material in the fractions was overcome by washing the magnesium silicate with water to remove soluble salt (largely sodium sulfate). The fraction overlap that resulted from long leading portions of peaks running into the long trailing portions of preceding peaks was traced to the presence of small amounts of water in ordinary reagent grade solvents. It became evident that the presence of small amounts of water introduced enough partition properties to bring about undesirable features and that magnesium silicate was most useful as a very dry (activated) adsorption column.

The trace of water in the system appears to be removed by 2,2-dimethoxypropane added to all of the solvents for chromatography, and is removed by treatment of the solvents with molecular sieve 5A. 2,2-Dimethoxypropane appears to react rapidly and virtually quantitatively with water under suitable conditions to yield methanol and acetone as reaction products. Neither of these reaction products interferes with the chromatographic separations under the conditions finally developed. Molecular sieve 5A is less desirable as some silicate can appear in column fractions (see below). Although dehydration eliminates the long leading portion of peaks, the trailing of peaks characteristic of adsorption chromatography is still present.

TABLE IV

Elution of Lipids from Magnesium Silicate (Florisil) Columns

Solvent	Substance
(1) Chloroform *	Cholesterol
(2) Chloroform/methanol, 19/1 *	Ceramide
(3) Chloroform/methanol, 2/1 *	Cerebroside + sulfatide
(4) Methanol *	Phosphatidyl ethanolamine + ganglioside
(5) Chloroform/methanol, 2/1 + 7% water	Lecithin + sphingomyelin + lysolecithin + phosphatidyl serine + inositol phosphatide

* 5% 2,2-dimethoxypropane added.

Table IV shows how a magnesium silicate column may be used for the separation of pure cholesterol, ceramide, and a mixture of cerebroside and cerebroside sulfate. Although phosphatidyl ethanolamine and ganglioside can be eluted with absolute methanol, and then lecithin, sphingomyelin, and phosphatidyl serine can be eluted together with chloroform/methanol containing water, these fractions are spread out over very large solvent volumes and a good deal of magnesium silicate is eluted along with lipid. Fairly accurate estimates of these latter two mixtures can be made if a second column is used as a control to determine the amount of adsorbent eluted with each solvent mixture. It was preferred to use other techniques for the recovery of these lipids however, as indicated elsewhere in this report.

The mixture of cerebroside and sulfatide eluted from magnesium silicate is separated on DEAE cellulose. Cerebroside is not adsorbed to DEAE while sulfatide is firmly adsorbed and must be eluted with a basic solvent. It is to be noted that a pure ceramide fraction is obtained from the magnesium silicate column and that the amount, although quite small as indicated in Table I, agrees closely with the amount obtained from silicic acid-silicate-water columns used in conjunction with DEAE column chromatography. Ceramide does not appear to have been isolated by previous investigators.

Cellulose Column Chromatography

As indicated in a report on paper chromatography of lipids (1), ganglioside can be chromatographed on unimpregnated filter paper with solvents containing water. Lea and Rhodes (19) and Smith (20) have used cellulose column chromatography for the removal of water soluble substances from lipid mixtures. Svennerholm (21) has described a procedure for the elution of cellulose columns to give a fraction containing ganglioside contaminated with phospholipid. It was decided to explore the use of cellulose for column chromatography and, although the work has been much more limited than for the other column materials described above, cellulose does appear to be useful for the recovery of gangliosides and water soluble nonlipid contaminants from lipid mixtures.

The cellulose must be washed with methanol and then with water to remove impurities. Washing is necessary with the best available grades of Whatman cellulose powder for chromatography. After drying,

the cellulose is slurried in chloroform and transferred to a chromatography tube to make a column 4.5 x 50 cm. packed under 2.5 psi nitrogen pressure. Approximately one-third of a column volume of a mixture of chloroform/methanol/water (16/4/1) is passed through the column, and the lipid sample (10 g.) is then applied in the same solvent mixture. Elution of total lipid minus ganglioside and water soluble nonlipids is accomplished with a mixture of chloroform/methanol/water, 16/4/1 (a two-phase mixture). When the complete elution of lipid beginning at the solvent front is assured by the absence of visible solid after evaporation of an aliquot of the chromatographic effluent, ganglioside is eluted with chloroform/methanol/water (4/15/1). When the solids test becomes negative again, water soluble nonlipids are eluted quantitatively with 10% water in methanol.

This procedure gives separations that are different from the ones described by Svennerholm (21). Ganglioside is not obtained in the first peak as found by Svennerholm. Ganglioside appears to be eluted entirely in the second fraction. The second fraction is not contaminated with phospholipid, and ganglioside is not obtained in the third peak in contrast to the findings of Svennerholm. The appearance of ganglioside in the first and third fractions described by Svennerholm may be related to overloading of the column and to a solvent change prior to complete elution of ganglioside. When columns were prepared in chloroform/ethanol/water, as described by Svennerholm, phosphatidyl serine lagged back into the ganglioside fraction, and it is probable that this accounts for the lipid contaminating his ganglioside fraction. The binding of phosphatidyl serine through water to cellulose hydroxyl groups is similar to the binding through water to silicate noted above. The cellulose column procedure can be used to check the values obtained for ganglioside and water soluble nonlipids by other methods.

Infrared Spectroscopy in Column Chromatography

Infrared is particularly useful as an aid in characterization. When a substance appears in the appropriate column chromatographic fraction and migrates on paper as a particular lipid class, infrared is the single most convenient means to confirm this tentative identification. In experience here, infrared examination is usually less sensitive than paper chromatography for the detection of minor impurities. It is particularly useful however as a rapid means for demonstrating the presence of various adsorbents in column fractions. Small amounts of many adsorbents that would not be expected to have any appreciable solubility in the organic solvents used for chromatography can appear in column effluents. The effluents may be clear and solids may pass through a filter. This was observed for such varied substances as molecular sieve 5A, calcium sulfate, magnesium silicate, silicic acid, and diethylaminoethyl (DEAE) cellulose "fines." When fractions containing these substances are evaporated to dryness, a solid is recovered that may be soluble in organic solvents. A particularly confusing type of behavior is that encountered with some preparations of DEAE cellulose. DEAE "fines" may appear to dissolve in or may disperse in a solvent and give the impression of being a moderately insoluble lipid material. The "fines" will give a strong ninhydrin test and char when heated. Infrared examination rapidly discloses the nature of such materials.

The use of infrared in determining the nature of extraneous materials in column fractions can be illustrated by two chromatographic runs carried out in this laboratory (columns 282 and 283). The columns were scaled up from the usual analytical runs. Column 282 was run with magnesium silicate as adsorbent. The column was 15.2 cm. i.d. by 10 cm. high, and 10 g. of total beef brain lipid was applied to the column in chloroform. Redistilled chloroform and methanol were used and both solvents were treated before mixing with molecular sieve 5A for dehydration. To insure dehydration a layer 2 in. high of anhydrous calcium sulfate (Drierite) was placed over the magnesium silicate bed after the sample had been applied.

The magnesium silicate column was eluted with chloroform (cholesterol fraction); chloroform/methanol, 19/1, (ceramide fraction); and chloroform/methanol, 2/1, (cerebroside + sulfatide fraction). The cerebroside plus sulfatide fraction from the magnesium silicate column was then passed through a DEAE cellulose column 15.2 cm. in diameter and 10 cm. high (column 283). The DEAE was found satisfactory for small scale analytical columns and was placed in the acetate form. Cerebroside was not adsorbed, while sulfatide was quantitatively adsorbed. Ordinarily the sulfatide was eluted with chloroform/methanol, 4/1, containing 20 ml./liter concentrated aqueous ammonia immediately after cerebroside was collected. In this case, however, the bed was first washed with methanol, water, and 5% aqueous ammonia and then with chloroform/methanol, 4/1, containing 20 ml./l. concentrated aqueous ammonia. The prewashing was done to remove silicate prior to elution of sulfatide. The chloroform/methanol/ammonia fraction did not have the solubility properties of the expected sulfatide and was found to be composed of DEAE "fines" by infrared examination (see Figs. 11,12). Evidently the prewashing with water and aqueous ammonia had changed the column characteristics so that sulfatide was not eluted normally and "fines" appeared in large amounts in contrast to the usual results. Subsequently the true sulfatide fraction was eluted with chloroform/methanol, 2/1, saturated with concentrated aqueous ammonia.

The spectrum of the small amount of solid remaining after evaporation of a forecut of chloroform collected prior to the time cholesterol began to appear in the effluent from the magnesium silicate column is shown in Figure 1, and the spectra from molecular sieve 5A and magnesium silicate are shown in Figures 2 and 3. Evidently the small amount (4.9 mg./500 ml.) of material in this forecut was silicate. This fraction also shows spectral characteristics not attributable to silicate. The absorption at 3.4 and 7.2 μ is characteristic of a trace of residue formed when redistilled chloroform or chloroform/methanol mixtures are evaporated to dryness. This typical trace material is shown in Figure 4.

The major cholesterol fraction gave the spectrum shown in Figure 5. No evidence of silicate can be observed as the amount of silicate is too low (1% or less). A small tail fraction (2 l. of solvent contained only 4.2 mg. of solid) gave a mixed spectrum (Figs. 6 and 7). Both cholesterol and silicate appeared to be present in the tailing fraction, and this was con-

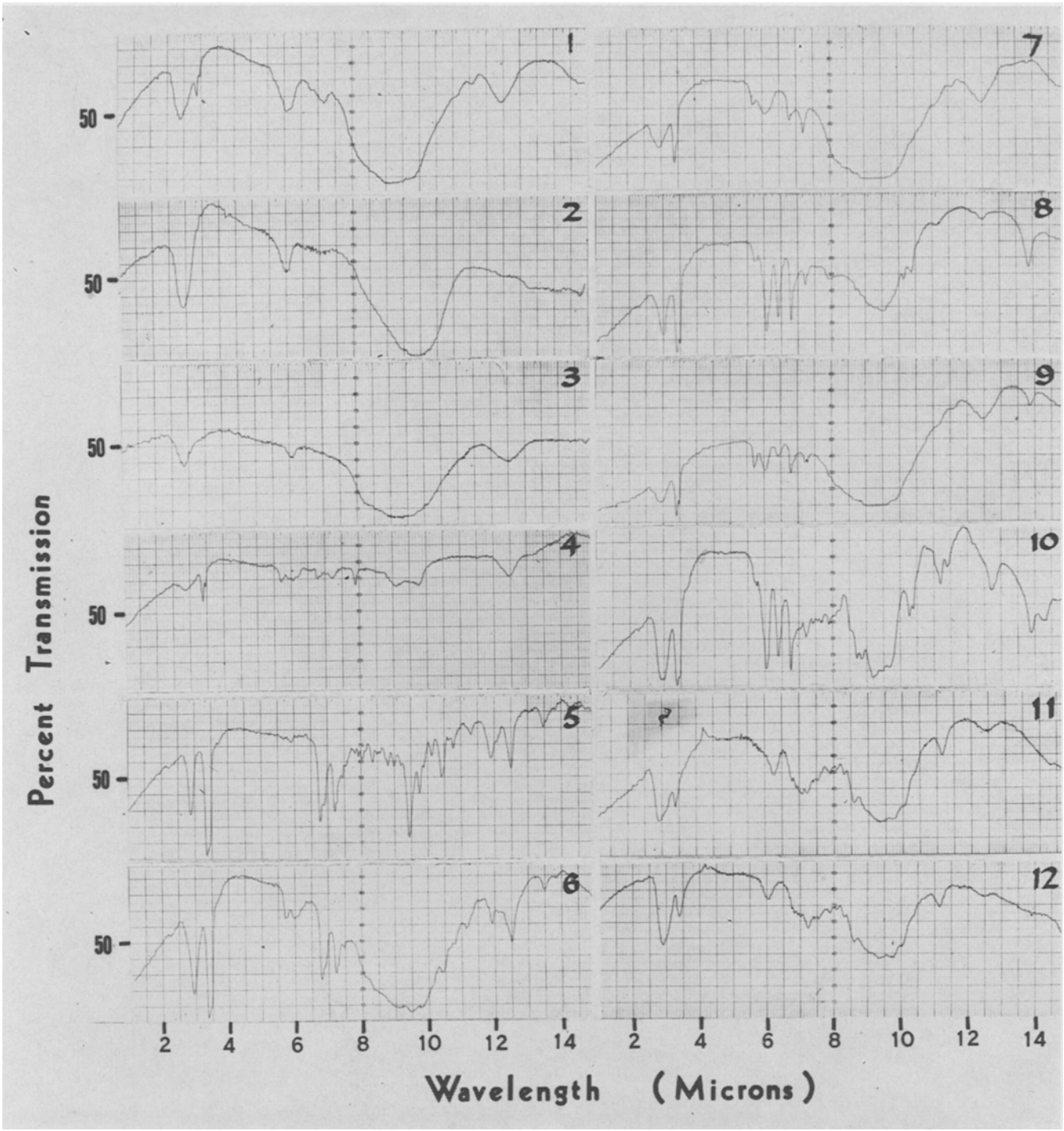


FIG. 1. Infrared spectrum of chloroform forecut prior to emergence of sample from a magnesium silicate column 282 to show trace of silicate and absorbing solvent residue that contaminated the fractions slightly. 2.1% sample pressed into a 0.14 mm. thick potassium bromide pellet. Compare with Figures 2 and 3. (See text for details.)

FIG. 2. Infrared spectrum of molecular sieve 5A used to dehydrate solvents for elution of column 282. 2.4% sample pressed into a 0.14 mm. thick potassium bromide pellet. Compare with Figures 1 and 3. (See text for details.)

FIG. 3. Infrared spectrum of magnesium silicate (Florisil) used as adsorbent for column 282. 2.6% sample pressed into a 0.17 mm. thick potassium bromide pellet. Compare with Figures 1 and 2. (See text for details.)

FIG. 4. Infrared spectrum of a residue left after evaporation of 500 ml. of redistilled chloroform/methanol, 19/1, collected as effluent from a control magnesium silicate column eluted as for column 282 without application of sample. Sample cast as a thin film from chloroform on a silver chloride plate. (See text for discussion.)

FIG. 5. Infrared spectrum of cholesterol fraction eluted from column 282. 4.1% sample pressed into a 0.15 mm. thick potassium bromide pellet. (See text for discussion.)

FIGS. 6 and 7. Infrared spectra of small tailing portions of the chloroform fraction from column 282 to show presence of silicate in this portion of the fraction. Samples (not weighed) pressed into potassium bromide pellets 0.15 mm. thick. (Compare with Figs. 3, 4, and 5.)

FIG. 8. Infrared spectrum of the main portion of the ceramide fraction from column 282. 2.0% sample pressed into 0.36 mm. thick potassium bromide pellet. Compare with Figure 9. (See text for details.)

FIG. 9. Infrared spectrum of very small tailing portion of the ceramide fraction from column 282. 1.9% sample pressed into 0.27 mm. thick potassium bromide pellet. Compare with Figures 3, 4, and 8. (See text for details.)

FIG. 10. Infrared spectrum of the cerebroside fraction from a DEAE cellulose column (283, see text for details). 3.0% sample pressed into 0.25 mm. thick potassium bromide pellet.

FIG. 11. Infrared spectrum of the "sulfatide" fraction from column 283 (see text). The fraction was actually "fines" of DEAE cellulose (compare with Fig. 12). 2.6% sample pressed into a 0.18 mm. thick potassium bromide pellet.

FIG. 12. Infrared spectrum of DEAE cellulose of type used as adsorbent for column 283 (see text for details). 4.3% sample pressed into potassium bromide. Compare with column 283 fraction shown in Figure 11.

firmed by paper chromatography. The solids test readily picked up the small amount (2 mg. or less/l.) of silicate in subsequent chloroform fractions. The cholesterol percentage was determined by weight and found to be 20.3%, a value in agreement with the value in Table I. Evidently the very small amount of inorganic material did not influence the determination.

The ceramide peak (eluted with chloroform/methanol, 19/1) was collected as one main fraction and a small tailoff portion. The tailing portion was evidently mainly silicate (Fig. 9). The spectrum of the ceramide (Fig. 8) was altered slightly by silicate because of the very small amount of lipid. A small absorption band at 5.72 μ is apparent. This is not due to the solvent residue noted above. The weight of ceramide corresponded to 0.35% of the total material applied, a value slightly higher than usually obtained (0.30) due to the presence of silicate.

The cerebroside spectrum shown in Figure 10 is typical. This lipid was recovered after passage through DEAE that removed silicate. The small absorption band in the ester carbonyl region is not from solvent residue.

Figure 11 is the spectrum prepared from the first "sulfatide" fraction referred to above that was eluted from the DEAE column with the first chloroform/methanol/aqueous ammonia mixture. It was essentially all DEAE "fines" as shown by comparison with the spectrum of DEAE (Fig. 12).

Acknowledgments

Richard Ray assisted in the preparation of the photographic illustrations and Nicholas Nicolaides and Rudolfo Paoletti made valuable contributions to the work on magnesium silicate column chromatography. The experimental work was supported in part by Grants C-3134 and B-1847 from U.S. Public Health Service and Research Grant No. DA-CML-18-108-61-G-7 from the U.S. Army Chemical Research and Development Laboratories, Army Chemical Center, Maryland.

REFERENCES

1. Rouser, George, Bauman, A.J., and Heller, D.J., J. Am. Oil Chemists' Soc., *38*, 565–581 (1961).
2. Rouser, George, O'Brien, J., and Heller, D., J. Am. Oil Chemists' Soc., *38*, 14–19 (1961).
3. Rouser, George, Bauman, A.J., and Kritchevsky, G., Am. J. Clin. Nutrition, *9*, 112–123 (1961).
4. Nicholas, H.J., Hiltibran, R.C., and Wadkins, C.L., Arch. Biochem. Biophys., *59*, 246–251 (1955).
5. Gale, P.H., Koniuszy, F.R., Page, A.C., Jr., and Folkers, K., J. Am. Chem. Soc. (in press).
6. Folch, J., J. Biol. Chem., *177*, 505–519 (1949).
7. Grado, C., and Ballou, C.E., J. Biol. Chem., *236*, 54–60 (1961).
8. Borgström, B., Acta Physiol. Scand., *25*, 101–110 (1952).
9. Barron, E.J., and Hanahan, D.J., J. Biol. Chem., *231*, 493–503 (1958).
10. Lea, C.H., Rhodes, D.N., and Stoll, R.D., Biochem. J., *60*, 353–363 (1955).
11. Lea, C.H., "Biochemical Problems of Lipids," Butterworths Scientific Publications, 1956, pp. 81–90.
12. Hanahan, D.J., Dittmer, J.C., and Warashina, E., J. Biol. Chem., *228*, 685–700 (1957).
13. Rouser, George, in Sphingolipidoses and Allied Diseases, Edited by B.W. Volk and S.M. Aronson, Academic Press (in press).
14. Rouser, George, Bauman, A.J., O'Brien, J., and Heller, D., Federation Proc., *19*, 233 (1960).
15. Phillips, G.B., Biochim. et Biophys. Acta, *29*, 594–602 (1958).
16. Marinetti, G.V., Erbland, J., and Stotz, E., Biochim. et Biophys. Acta, *30*, 41–43 (1958).
17. Radin, N.S., Lavin, F.B., and Brown, J.R., J. Biol. Chem., *217*, 789–796 (1955).
18. Kishimoto, Y., and Radin, N.S., J. Lipid Res., *1*, 72–78 (1959).
19. Lea, C.H., and Rhodes, D.N., Biochem. J., *54*, 467–469 (1953).
20. Smith, R.H., Biochem. J., *57*, 130–139 (1954).
21. Svennerholm, L., Nature, *177*, 524–525 (1956).

Column Chromatography of Fatty Acids[1]

HERMANN SCHLENK and JOANNE L. GELLERMAN, The Hormel Institute, University of Minnesota, Austin, Minnesota

IN PART I, column chromatography of fatty acids is discussed, with emphasis being placed on practical aspects. Examples from the literature are quoted.

In Part II, experimental details are given of a new liquid-liquid chromatographic system and its use in isolating minor component fatty acids of menhaden oil.

The value of column chromatography of fatty acids depends on its combination with other analytical procedures.

Chromatography of fatty acids in liquid phase over a solid or another liquid phase is now used mainly for preparative purposes rather than for immediate quantitative analyses. The ultimate goal, of course, may still be analytical. Some circumstances that may favor the use of LC[2] rather than other methods of separation are pointed out in the following:

Amounts

Several grams of material can be chromatographed, depending upon the complexity of the mixture, for isolation and enrichment of components.

Examples. LSC and LLC are essential steps in a current preparation of methyl arachidonate (1). Individual fatty acids of rat lipids have been isolated by means of LLC in amounts large enough for determining their radioactivity in conjunction with chemical degradation (2). Odd-numbered (3) and other minor fatty acids of fish oils have been enriched by LLC so that other separation procedures could then be utilized for their isolation and ultimate identification (see Part II).

Instability

The temperature at which LC is carried out does not alter the structure of labile compounds. Although

[1] Work presented in Part II was supported by grant RG-4226 from the Division of General Medical Sciences of the U.S. Public Health Service, and by the Hormel Foundation.

[2] Abbreviations used in this article are: LC = liquid chromatography, i.e., LSC = liquid-solid (adsorption) chromatography, and LLC = liquid-liquid (partition) chromatography; PC = paper chromatography; GLC = gas-liquid chromatography.