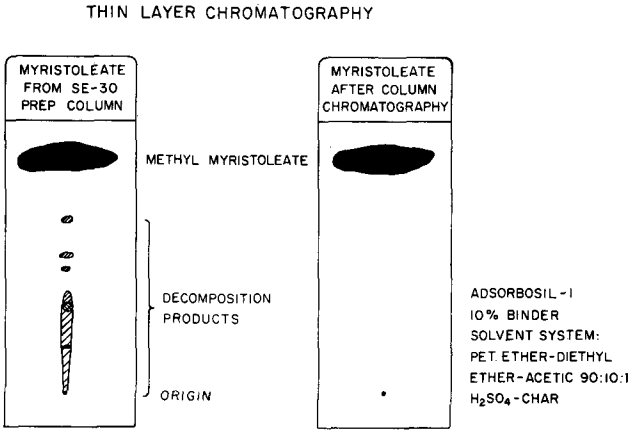

FIG. 13. TLC analysis of methyl myristoleate before and after silica gel treatment.

for methyl myristate and component A broadened appreciably until they finally merged into one shoulder on the front of the myristoleate peak.

It is not clear whether the relatively small samples are required because the stationary phases (polyesters) are easily overloaded or whether the separations performed were so critical that near optimum conditions were required.

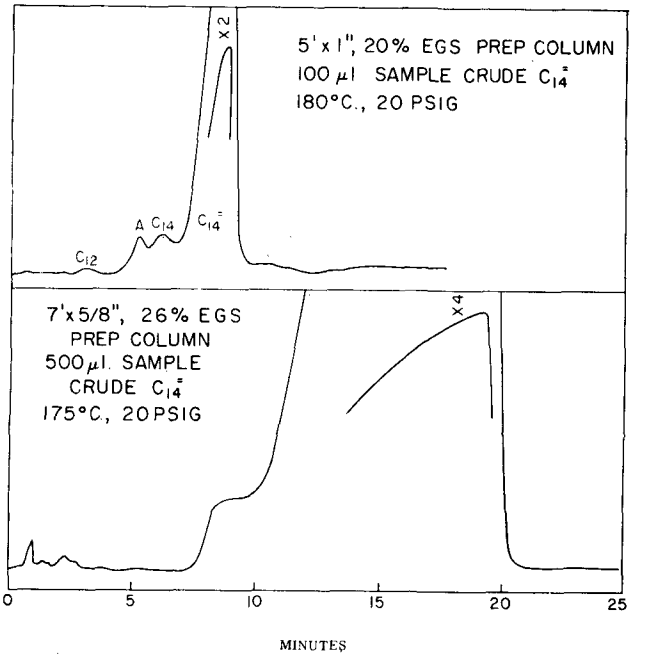

FIG. 14. Comparison of GLC traces from preparative runs without severe overloading (top) and with overloading (bottom).

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Lipid Composition of Beef Brain, Beef Liver, and the Sea Anemone: Two Approaches to Quantitative Fractionation of Complex Lipid Mixtures

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Abstract

Two new schemes for fractionation of complex lipid mixtures are presented. Their use for the study of lipids of beef brain, beef liver, and the sea anemone are described. Apparatus and techniques for working in an inert atmosphere, evaporation of solutions in the cold under nitrogen, use of infrared spectroscopy for examination of lipids and their hydrolysis products, preparation and elution of diethylaminoethyl (DEAE) cellulose and silicic acid-silicate columns, and general column combinations that can be used to fractionate complex lipid mixtures are considered in detail. The first scheme, employing DEAE cellulose columns followed by thin layer and paper chromatographic examination of the fractions, was applied to liver lipids. The many components, some of them new lipids not previously detected, are clearly seen with this technique but are not seen when paper or thin layer chromatography alone or silicic acid chromatography are used.

The second scheme employing DEAE for initial fractionation, followed by complete separation on silicic acid and silicic acid-silicate columns, was applied to lipids of the sea anemone and beef brain. Typical lecithin and phosphatidyl ethanolamine were isolated, but sphingomyelin was not found. A new sphingolipid, ceramide aminoethylphosphonate, with a free amino group and a direct carbon to phosphorus bond was isolated and characterized. The methods used for quantitative isolation, the infrared spectra, and the amounts of cholesterol, ceramide, cerebroside, galactosylglyceride, sulfatide, sphingomyelin, lecithin, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, triphosphoinositide, phosphatidic acid, cardiolipin, and ganglioside of beef brain are presented. Finally, the types of lipid-nonlipid interactions disclosed by column chromatography and their potential application to biological problems are discussed.

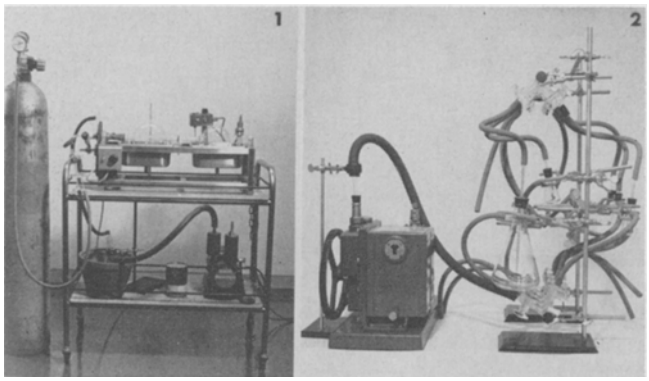


FIG. 1. Rotary (flash) evaporator. See text for details.
FIG. 2. Vacuum rack. See text for details.

Introduction

INTEREST IN THE functions of lipids in complex biological systems (1–4) has led us to extensive and detailed studies of lipid chemistry and improved methods for the isolation and characterization of lipids. This report presents methods useful for the quantitative fractionation of complex lipid mixtures and features two general fractionation schemes. Both schemes depend upon the high resolving power of DEAE (diethylaminoethyl) cellulose columns. The first scheme employs DEAE cellulose columns followed by examination of the fractions by paper (5) and thin layer chromatography (TLC). The second scheme, an extension of the first, employs DEAE for initial fractionation followed by complete separation on other columns (6).

When a lipid mixture is examined for the first time, the first scheme is most useful since it offers a rapid means for defining the complexity of the mixture. Some pure lipid classes can be eluted from the DEAE column, and the complexity of other fractions can be judged by paper and TLC. The method serves as a basis for the selection of the most interesting fractions for more extensive study and the amount of a particular phospholipid in a fraction can be estimated by determination of phosphorus in spots after paper or TLC. The second scheme is necessary for quantitative determination and careful characterization of all components of a complex mixture and precise determination of the fatty acid and fatty aldehyde composition of individual lipid classes.

General Laboratory Techniques in Lipid Chemistry

The necessity for working in an inert atmosphere (e.g. nitrogen) in quantitative lipid studies has been stressed and some simple ways of performing laboratory operations under nitrogen have been described (6). A useful new addition to laboratory supplies for working in an inert atmosphere is the glove bag (Model 3X, available from the I²R Company, Cheltenham, Pa.). These plastic bags have plastic gloves sealed in and can be used as inexpensive, disposable dry boxes for many laboratory manipulations. If they become contaminated or damaged, they can be replaced at nominal cost.

Nitrogen lines can be installed with plastic tubing (Polyflow, Imperial Brass Mfg. Co., Chicago, Ill.) to replace the usual copper lines. We use ¼ in. tubing. The tubing is very flexible, withstands high pressures (up to 180 lb/in²), can be cut with scissors or knife, and does not leak. It is inexpensive and comes in several colors that facilitate recognition of separate lines. A variety of fittings (Imperial and Swagelok) are available and can be placed readily by hand in a few seconds (and be leak proof). It is convenient to have spools of several hundred feet available. Within minutes a line for nitrogen or other gas (as for gas chromatography) can be installed as needed.

Evaporation of solutions. Careful work requires redistilled solvents. Distillation removes a small solid residue present in reagent grade solvents. This residue can interfere with quantitative isolation procedures and with the characterization of minor components of lipid mixtures since the solvent residue may be a significant portion of the total weight of material.

We utilize two evaporation devices for concentrating solutions. A conventional rotary type evaporator is recommended for large volumes or when a relatively small number of fractions are to evaporated quickly. Figure 1 illustrates our apparatus. It consists of a Flash Evaporator (Buchler Instruments Inc., New York, N. Y.) with the trap cooled in methyl cellosolve-dry ice and a small auxiliary trap cooled in the same mixture. Reduced pressure is provided by a vacuum pump of good capacity. The Langdon pump (Hevi-Duty Electric Co., Milwaukee 1, Wisconsin) is ideal for this purpose. The pump operates on 50 ml of ordinary 30 wt motor oil that is replaced after about 8 hr of operation. The standard taper joints of the evaporator do not require lubrication, and grease should not be used in lipid work since fractions can become contaminated. A small manometer is mounted in the line to facilitate detection of leaks in the vacuum system and return of the flask to atmospheric pressure with pure nitrogen.

The system is thoroughly flushed with nitrogen and evaporation is begun by allowing the solution to cool very rapidly to about 0C (or below). These low temperatures are maintained by allowing the evaporator flask to rotate in air rather than in the water bath. If a large cake of ice develops on the sample flask, it is removed by pouring water over the flask or by adding enough water to the bath to bring the temperature of the outside of the flask to slightly above 0C. When glacial acetic acid is evaporated, the water bath is maintained at 1 or 2° above the freezing point of the solvent by carefully adjusting the water level of the bath so that a small portion of the flask dips into the water. Evaporation of 500 ml or more of most solvents can be accomplished in 30 min or less at low temperature and under nitrogen. An efficient vacuum pump makes this possible. Vacuum pumps are far superior to water pumps since they allow the efficient use of low temperatures. To prevent vigorous boiling and loss of material at the reduced pressure, solvent must be evaporated at a low temperature when a vacuum pump is used.

After the bulk solutions have been evaporated to dryness in the rotary evaporator, it is usually desirable to transfer the lipid to a suitable vessel for weighing. The lipid is transferred to a preweighed side-arm filtering flask of the appropriate size (25–125 ml capacity) and placed on a vacuum rack (Fig. 2). The flasks are connected to a vented vacuum pump (Model 1402B, W. M. Welch Scientific Co., Chicago, Ill.) and fitted with rubber stoppers into which the

ends of small (0.1 ml) pipettes or capillary tubes are inserted for the introduction of pure nitrogen. It is helpful if the holes in the stoppers are just large enough to allow the nitrogen inlet tube to be pushed closer to the surface of the liquid during evaporation. Evaporation takes place more rapidly with the inlet tube close to the liquid level. The flask contents are first exposed to partial pressure reduction of the vacuum pump (stopcock partially opened) with a fast flow of nitrogen for rapid cooling to or below 0C. The cold flasks are then exposed to the full capacity of the vacuum pump (stopcocks to the vacuum line wide open) and a small steady stream of nitrogen is passed over the solvent surface. In this way it is possible to evaporate solvent with a cake of ice around the flask and under nitrogen. Manifolds in use in our laboratory have been prepared with ten 6 mm stopcocks (preferably of the pressure type) and open ends fitted with rubber stoppers to facilitate cleaning. If small volumes are used for transfers, evaporation of the solvent may be accomplished in 5–10 min. Even bulk fractions of several hundred milliliters can be concentrated with this apparatus in 1–3 hr. Acetic acid solutions can be evaporated after addition of chloroform and/or methanol to prevent freezing. Aqueous solutions or dispersions are usually lyophilized by conventional techniques or with a rotary evaporator with the simple flask rotating in air.

When a large amount of lipid is to be recovered, and particularly when it must be mixed owing to layering in an uneven manner duing evaporation, a vacuum desiccator is substituted for the side arm flasks. The desiccator is fitted with a nitrogen inlet tube and a glass tube for connection to the vacuum rack. A thick slurry of the lipid is transferred to a large glass mortar and covered with aluminum foil. The nitrogen inlet tube is passed through a hole in the foil. The foil serves to prevent loss from bumping, and, in the all glass system, the flow of nitrogen can be regulated with ease since the surface of the liquid is clearly visible. In some cases a bell jar is more convenient and can be used for the same purpose.

A word of caution is necessary concerning the use of rubber stoppers. Black rubber stoppers are convenient and can be used successfully in routine operation without introduction of color or weighable impurities when some simple precautions are followed. Stoppers should be perfectly smooth (to avoid contamination of the sample with small bits of rubber dislodged mechanically) and should not be placed in a flask containing solvent (particularly chloroform) for more than 5–10 min unless the sample is on the vacuum rack. When not under vacuum, the solvent may extract colored material from the stopper. The stoppers with the nitrogen inlet tubes inserted can be cleaned with a good detergent.

Removal of salt from fractions. The use of solvents containing salt for the elution of DEAE cellulose columns is described below. Quantitative separation of lipid from salt requires considerable care. We recommend the use of ammonium acetate (rather than potassium acetate originally used) (6) since ammonium acetate can be removed during evaporation of solvent or by lyophilization. Both potassium and ammonium acetates are quite soluble in chloroform/methanol mixtures. When a chloroform/methanol mixture containing less than 0.01 M ammonium acetate is evaporated on the rotary evaporator, the salt is usually removed completely along with the solvent. Some salt usually remains with the lipid at concentrations above 0.01 M. When the original concentration of salt is not above about 0.05 M, its complete removal from the lipid can frequently be accomplished quickly by treating the solids with a small amount of chloroform/methanol 2/1, adding enough water to produce a slight turbidity, and re-evaporating. This may be repeated several times until salt crystals are no longer visible.

At very high salt concentrations (0.1–0.4 M) it is usually desirable to extract the lipid from most of the salt with chloroform or chloroform/methanol 98/2, filter through a prewashed glass wool plug, and remove any residual salt on the rotary evaporator after treatment with chloroform/methanol/water.

A small amount of residual ammonium acetate can be removed from lipid samples by treating with water, freezing, and drying from the frozen state. Lyophilization can be carried out on a rotary evaporator and is a convenient technique to use as a check for salt in samples after weighing. Samples in 50 ml side arm flasks can be treated with 1–5 ml of water and frozen. The flask is fitted with a rubber stopper and attached by the side arm to a lyophilization apparatus and dried. If the sample weight remains constant after lyophilization, the lipid is almost certainly free of ammonium acetate.

Extraction of Lipids

Quantitative studies of lipid composition require quantitative extraction procedures. Although many different solvent mixtures have been used, we prefer chloroform/methanol 2/1 as recommended by Folch et al. (7). Most lipids are relatively soluble in the mixture and it has been used extensively in many laboratories. The preparation of a lipid extract can be divided into four steps: 1) extraction of fresh tissue, 2) solvent removal, 3) re-extraction of dry lipid (as with chloroform/methanol, chloroform, or other solvent) to leave behind some nonlipid material, and 4) final evaporation, mixing, and drying. Some procedures utilize a water wash to free the extract of nonlipid impurities. Because lipid may be lost in such procedures, this step is not used in this laboratory.

Extraction of brain lipids. Fresh wet brain or a frozen sample is first extracted three times at room temperature with chloroform/methanol 2/1 as previously described (6,8). The first extraction is carried out with 20 ml of solvent per g wet weight of tissue, and the residue is extracted two times with 10 ml of solvent per g wet weight of fresh tissue. The entire procedure is carried out under an atmosphere of pure nitrogen and with redistilled solvents that have been deaerated. Solvent is removed by evaporation in a rotary evaporator under nitrogen with the sample maintained near or below 0C as described above. It is important to have a two phase system during evaporation of solvent to insure denaturation of protein present in the extract. The original crude lipid extract that contains denatured protein is then thoroughly dried in a vacuum desiccator over KOH (all operations to this point are carried out in the same round bottom rotary evaporator flask, usually of 2-liter capacity). The crude lipid is then extracted with three portions of chloroform/methanol 2/1. For extraction of lipid from one beef brain weighing approximately 400 g, three one-liter portions of

TABLE I

Paper Chromatographic Methods

	Solvents	Conditions *	Substances
(18)	Chloroform	1, 60 min	Dinitrophenyl derivatives of sphingosine and allied compounds
(19)	Carbon tetrachloride	1, 120 min	α,β,γ,Δ tocopherols
(20)	Chloroform/methanol 98/2	5, 60 min	Sphingosine, phytosphingosine and allied compounds
(21)	Chloroform/methanol 95/5	5, 60 min	Sphingosine, phytosphingosine and allied compounds
(22)	Chloroform/methanol 2/1	2,3,4, or 5, 60 min	Phospholipids, sulfatides, and cerebrosides
	(a) plus 10 ml H_2O/liter		
	(b) plus 20 ml H_2O/liter		
	(c) plus 40 ml H_2O/liter		
(23)	Chloroform/methanol 3/1	2,3,4, or 5, 60 min	Phospholipids, sulfatides, and cerebrosides
	(a) plus 10 ml H_2O/liter		
	(b) plus 20 ml H_2O/liter		
	(c) plus 40 ml H_2O/liter		
(24)	Chloroform/methanol 4/1	2,3,4, or 5, 60 min	Phospholipids, sulfatides, and cerebrosides
	(a) plus 10 ml H_2O/liter		
	(b) plus 20 ml H_2O/liter		
	(c) plus 30 ml H_2O/liter		

* 1) Acidic silicic acid paper (prepared as in reference 5), ascending technique, chamber lined with paper saturated with solvent just prior to insertion of papers.
2) 0.05 N alkaline silicic acid paper, ascending, unlined chamber.
3) 0.1 N alkaline silicic acid paper, ascending, unlined chamber.
4) 0.25 N alkaline silicic acid paper, ascending, unlined chamber.
5) 0.5 N alkaline silicic acid paper, ascending, unlined chamber.
The number of minutes designates the time required for solvent to travel 8 to 10 in. at 25 ± 1C.

chloroform/methanol 2/1 are used. The insoluble residue is removed by filtration through a sintered glass filter (medium porosity) and the residue containing denatured protein is discarded. If the residue is finely divided and tends to pass through the filter, the lipid should be recovered again from a chloroform/methanol/water solution, dried, and reextracted as before.

The operations during reextraction are conducted under nitrogen and evaporations are carried out as for the original extract in the rotary evaporator until the volume is reduced to 50–100 ml of thick suspension. The flask contents are then transferred to a large glass mortar and the remaining solvent removed in a vacuum desiccator under reduced pressure and a stream of nitrogen as described above. The lipid is then thoroughly dried over KOH, transferred to a glove bag (see above) and mixed thoroughly (under nitrogen) by scraping from the sides of the glass mortar with a spatula followed by pressing against the sides of the mortar with a glass pestle. This operation is repeated several times after which the lipid is transferred to weighed tubes and sealed under nitrogen. The lipid remaining on the mortar, pestle, and spatula is removed with chloroform/methanol 2/1, the solvent removed by evaporation in a preweighed flask, the lipid dried over KOH and weighed. This weight is then added to the total weight of sampled sealed in tubes to give the total quantity of lipid extracted with chloroform/methanol 2/1.

This procedure works well for the lipid (about 45 g) from one beef brain weighing about 400 g. Mixing in a mortar is necessary for brain lipids since different lipids are deposited at different rates during evaporation. This behavior is not observed with lipid mixtures from all sources.

The original tissue residue left after chloroform/methanol 2/1 extraction still contains a small amount of lipid. After the residue has been thoroughly freed of solvent and water by careful drying over KOH, it is extracted at room temperature with chloroform/methanol 7/1 saturated with concentrated aqueous ammonia (about 5.5%, v/v). We use three extractions (100 ml each) of the residue obtained from one beef brain weighing approximately 400 g. After the third extraction no further material is obtained. From one beef brain weighing about 400 g, 160–275 mg of solids is obtained. The operations are conducted under a nitrogen atmosphere and solvent is removed under nitrogen at reduced pressure in the cold.

The chloroform/methanol/ammonia extraction procedure has been applied to the residue obtained from beef heart mitochondria after exhaustive extraction with chloroform/methanol 2/1. The extract in this case was almost pure cardiolipin (ca. 10% of the total amount in mitochondria).

The chloroform/methanol/ammonia extraction procedure is superior to procedures using acidified chloroform/methanol. Chloroform/methanol/ammonia has been compared to chloroform/methanol/concentrated HCl (200/100/1) and chloroform/methanol/glacial acetic acid/water (4/2/1/0.5). Chloroform/methanol acidified with HCl extracts a great deal of pigment and substances that are relatively insoluble after evaporation of the solvent. Chloroform/methanol acidified with acetic acid extracts less nonlipid material, but the amount is still high. Chloroform/methanol/ammonia gives an extract that is almost colorless (entirely free of red pigment) and the solids dissolve readily and completely in chloroform/methanol mixtures, particularly if 1–2% water is added.

The water content of whole beef brain determined by drying to constant weight over KOH in a vacuum desiccator evacuated with a vacuum pump has been found to vary between 76.5 and 77.8%. The total crude lipid (that contains about 10% water soluble nonlipids as contaminants) has been observed to vary from 11.4–12.8% of the fresh weight. Nearly one-half of the total solids of beef brain is lipid.

Extraction of other tissues. Extraction of lipids from sources other than brain can be accomplished by modified techniques. It is desirable in some cases to perform reextraction of the original crude lipid (extracted from the tissue with chloroform/methanol 2/1) with a solvent other than chloroform/methanol 2/1. Both chloroform and petroleum ether (or hexane) have been used for this purpose. The hydrocarbon solvents are generally less satisfactory for quantitative work since some lipid may be left undissolved and water soluble nonlipids may be carried into the solvent phase. Chloroform has better solubility properties and is more satisfactory. We have used this solvent successfully in the extraction of lipids from the sea anemone where it possesses the advantage of leaving as an insoluble residue a great deal of salt originally extracted with chloroform/methanol 2/1. In this case it was shown by extraction of the chloroform insoluble residue with chloroform/methanol and paper chromatographic examination of the extract that no significant amount of lipid remained after chloroform reextraction. This test should be applied when chloroform or hydrocarbon solvents are used for reextraction.

Paper and Thin Layer Chromatographic Methods

Silicic acid impregnated paper can be divided into three general types: acidic, neutral, and alkaline.

These papers differ in silicate content. The acidic papers have a low silicate content, while the neutral papers have an appreciable silicate content. These two types of papers have been described previously (5). Various alkaline silicic acid papers that contain progressively increasing amounts of silicate have been added. Additional separations can be obtained with these new alkaline papers.

The chromatographic systems listed in Table I are numbered in sequence with those previously presented (5). System 18 utilizes acidic paper with chloroform as solvent and is useful for the separation of the dinitrophenyl derivatives of sphingosine and allied substances (Fig. 3). We have used this system in studies of sphingolipid hydrolysis products.

Systems 20–24 are those utilizing alkaline papers. There are three general variables in the alkaline paper systems: the amount of silicate, the chloroform/methanol ratio, and the amount of water. The amount of silicate in the system is controlled by the strength of the alkali used in preparing the paper. The alkaline papers are prepared from acid paper by dipping into 0.05, 0.1, 0.25, or 0.5N NaOH just long enough for the paper to be completely wet, removed quickly and air dried. This gives papers with increasing quantities of silicate and markedly different separation characteristics. Very good results can be obtained with alkaline papers without the addition of water to the solvent and when the methanol content of such systems is gradually increased the migration of lipids is likewise increased. When water is added to the system, some lipids are retained more tenaciously by the stationary phase while others are bound less firmly. There is thus a change in the order of migration of lipids with chloroform/methanol/water mixtures compared to chloroform/methanol mixtures.

Systems 20 and 21 are very useful for the separation of sphingosine, dihydrosphingosine, and phytosphingosine. This is illustrated in Figures 41 and 42 where the control substances were used along with an hydrolysate of a lipid isolated from the sea anemone. Systems 20 and 21 have disclosed that there are a number of components in acid hydrolysates of sphingolipids. These appear to be related to the presence of substances similar to sphingosine, dihydrosphingosine, and phytosphingosine, but differing in chain length and unsaturation. The erythro and threo forms of these bases are also separable in systems 20 and 21, and both forms can be visualized on paper chromatograms from acid hydrolysates. The remaining solvent systems (22–24) are particularly useful for the paper chromatographic separation of certain phospholipids and sulfatides.

Rhodamine 6G is an exceptionally sensitive stain for lipids on alkaline paper and photographic reproduction is excellent. Less than 1 μg of most of the lipids can be detected as a definite spot after paper chromatography. Since the background after staining is a deep purple, these chromatograms can be reproduced readily by photography under ultraviolet light with the Polaroid camera (5).

A TLC technique similar to that described by Habermann et al. (9) has been used in this laboratory and is still under study. Plates have been spread with the conventional spreader obtained from Brinckmann Instruments Inc., Great Neck, L. I., N. Y., using silica gel G obtained from Research Specialties Co., Richmond, California. The plates have been heat activated at 120C for 10–20 min, cooled in air for approximately 30 min, spotted as a single spot or as a row of very small spots (to give bar shaped spots on the final chromatogram), developed in chambers with or without a paper liner saturated with solvent, and sprayed with various reagents.

The most sensitive reagent that we have used is a modification of the Rhodamine 6G staining technique

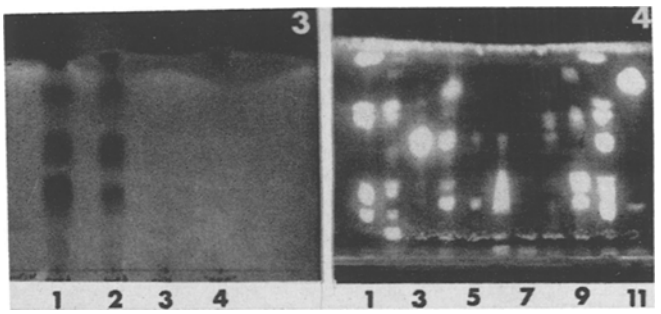


FIG. 3. Paper chromatogram (system 18, Table 1) of dinitrophenyl derivatives of sphingosine and allied substances. Spots 1 and 2, prepared from 30 μg of crude preparations, contain O-methyl sphingosine, dihydrosphingosine, and sphingosine (both erythro and threo forms). Spots 3 and 4 from dinitrofluorobenzene and dinitrophenol, demonstrate that spots are not seen in the body of the chromatogram that might be confused with dinitrophenyl derivatives.

FIG. 4. Thin layer plate developed with chloroform/methanol/water (65/25/4, v/v/v) and sprayed with alkaline Rhodamine 6G reagent as described in the text. The chromatographic results illustrate the findings with fractions from an overloaded DEAE column with brain lipid as sample (see text section on DEAE columns for details of elution). Acidic lipids are not retained completely and appear in more than one fraction. 100 μg of each fraction was applied and fractions are in order of elution (from left to right). Spot 1 shows lipid eluted with chloroform/methanol 9/1. The substances from above down are: cholesterol, 3 spots of cerebroside, lecithin, and sphingomyelin. Spot 2 shows a series of substances eluted with chloroform/methanol 9/1 after the lipids shown in spot 1 were eluted. Acidic lipids that should have been retained are present. From above down the components are cholesterol, uncharacterized acidic lipid, 3 spots of cerebroside, phosphatidyl ethanolamine, sulfatide, lecithin, sphingomyelin, and one ganglioside component. Spot 3, prepared from the main fraction eluted with chloroform/methanol 7/3, contains in addition to phosphatidyl ethanolamine (the major spot) small amounts of uncharacterized acidic lipids migrating ahead of the main spot and sulfatide and phosphatidyl inositol migrating behind the main spot. Spot 4 prepared from the methanol eluate (after eluting with chloroform/methanol 7/3) shows a large amount of acidic lipid that is normally not eluted with this solvent. The substances from above down are uncharacterized acidic lipid (2 major and 2 minor spots), sulfatide and phosphatidyl inositol. Spot 5 shows the tailing portion of the methanol eluate (primarily water soluble nonlipid materials) containing traces of acidic lipid. Spot 6 prepared from the acetic acid eluate (after methanol) composed primarily of phosphatidyl serine (major spot) and gangliosides migrating behind phosphatidyl serine. Spot 7 from substances eluted with methanol after glacial acetic acid showing only traces of ganglioside that were an insignificant part of the weight of the entire sample (about 0.2%). Spot 8 prepared from a very small fraction (eluted with chloroform/methanol 4/1 containing 20 ml of concentrated aqueous ammonia per liter) shows only traces of acidic lipids. Spot 9 is from the lipids eluted with the same solvent as in spot 8 with 0.1M ammonium acetate added to increase the eluting power. From above downward the lipids are cardiolipin, uncharacterized acidic lipid, sulfatide, and phosphatidyl inositol. Spot 10, from total beef brain lipid, shows from above down: cholesterol, three spots of cerebroside, phosphatidyl ethanolamine, and a complex area in which sulfatide, lecithin, phosphatidyl inositol, phosphatidyl serine, and sphingomyelin migrate without separation. Spot 11 is from cardiolipin isolated from beef heart mitochondria by column chromatography on DEAE. The apparent complexity of the lipid mixture judged by the composition of the fractions from an overloaded column is misleading since acidic components are not retained properly and are eluted with solvents other than those indicated in the text.

described previously for paper chromatograms (5). A mixture of 0.0025% Rhodamine 6G in 2 N NaOH (mixed a few seconds before use) is sprayed on the plate. Best results are obtained when solvent has been removed completely from the plate before spraying. If the drying time is too short, the purple background is not as intense and the yellow or orange spots are not as readily visualized. When the plate is allowed to stand for a short time after spraying and viewed under ultraviolet light, a point of maximum brightness is reached. The background becomes a deep purple or bluish-purple and the spots become a very bright yellow or orange. Other very useful general detection reagents are sulfuric acid-potassium dichromate (10,11), ammonium molybdate-perchloric acid (12), and phosphomolybdate (13). These reagents give spots visible under ordinary light that are readily reproduced with the Polaroid camera.

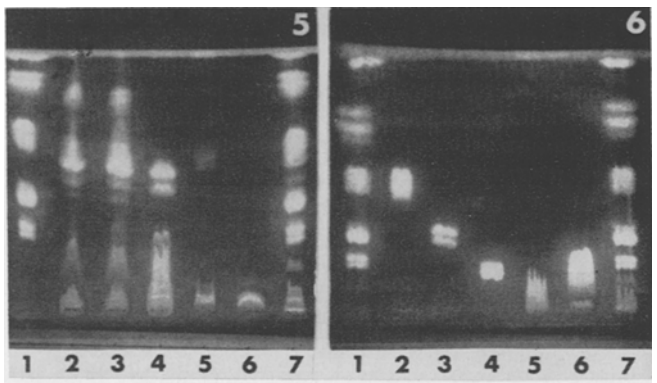


FIG. 5. A thin layer plate to illustrate division of a lipid mixture into nonacidic and acidic fractions, respectively (see text, elution of DEAE columns). The plate was developed with the same solvent as in Figure 4 (see text for details). The DEAE column was prepared in methanol, charged with brain lipid, and eluted with methanol followed by chloroform/methanol/ammonia containing 0.1 M ammonium acetate. Spot 1 was prepared from the methanol eluate. The components in spot 1 are from above downward: cholesterol (at the solvent front and difficult to distinguish from colored material at the solvent front), 3 incompletely separated spots of cerebroside, phosphatidyl ethanolamine, lecithin, and sphingomyelin. The latter separated almost completely into 2 separate spots presumably on the basis of fatty acid content. Spots 2, 3, and 4 were prepared from samples obtained at the first, middle and latter portion of the chloroform/methanol/ammonia/ammonium acetate eluate to show that some acidic lipids are eluted less readily than others. Spot 2 shows from above down: uncharacterized acidic lipids with a small amount of sulfatide, phosphatidyl inositol, and ganglioside. Spot 3 shows similar general composition to spot 2, while spot 4 shows from above down 2 spots of sulfatide (in this case sulfatide migrated well ahead of lecithin) and a mixture of components just off the origin including phosphatidyl serine, phosphatidyl inositol, and ganglioside. Ganglioside was eluted slowly with chloroform/methanol/ammonia/ammonium acetate so a change to acetic acid was made and spots 5 and 6 show ganglioside eluted with acetic acid. Spot 7 was prepared from whole beef brain lipid showing, in contrast to the spot of whole beef brain lipid in Figure 4, a relatively wide separation of a number of the brain lipids related to slightly different methods of heat activation of the plates and different equilibrium conditions inside the chromatographic chambers. From above down the components are: cholesterol, a trace spot from a mixture of ceramide and cardiolipin (barely detectible), 3 spots of cerebroside clearly seen, phosphatidyl ethanolamine, followed by 1 spot of sulfatide just touching the end of the phosphatidyl ethanolamine spot and a second sulfatide spot (clearly separated from surrounding spots), lecithin, sphingomyelin, a completely separated phosphatidyl inositol spot, with phosphatidyl serine and ganglioside admixed and just off the origin.

FIG. 6. Thin layer chromatogram prepared as described for Figure 4 showing in spot 1 from above down: chloesterol, three cerebroside spots, phosphatidyl ethanolamine, lecithin, and sphingomyelin; spot 2, phosphatidyl ethanolamine; spot 3, brain sulfatide (2 spots); spot 4, sphingomyelin; spot 5, phosphatidyl serine; spot 6, phosphatidyl inositol; and spot 7, whole beef brain lipid. Note the marked difference in sulfatide migration compared to Figure 5. Even relatively small changes in heat activation of the plates and equilibration conditions within the chambers may cause shifts in relative migration of acidic lipids (see text for further details).

We have encountered a wide degree of variability with TLC and conclude that the technique must be used with great care. Aside from variations in the migration of lipids on plates spread with different amounts of adsorbent, we have obtained different results with different preparations of silica gel. Marked variations have been observed depending upon the method of heat activation and cooling of the plate, the method of maintaining saturation in the chamber (whether or not a solvent saturated liner is used, exactly how the liner is saturated, and whether 1 or 2 plates are placed in the chamber), and the temperature of the run. One of the undesirable features of the variability of the TLC technique with chloroform/methanol/water mixtures is that the relative orders of migration of some lipids may be changed. With the same solvent system and the same silica gel preparation, acidic lipids have been observed to move to different positions relative to nonacidic lipids depending upon the equilibration conditions and the exact manner of treating the plate. Specifically it has been observed that the relative order of migration of phosphatidyl serine and phosphatidyl inositol with respect to sphingomyelin can be changed and that sulfatide may migrate either well ahead of or overlap with the lecithin spot (Fig. 4,5,6). Authentic substances should be used as standards on each plate.

Aside from the variability noted above, TLC may lead to deceptive results seldom observed with paper chromatography. Generally speaking, paper chromatography can be used to recognize individual lipid classes without separation into more than one spot of the various members of the lipid class containing different fatty acids. We have found that most lipid classes tend to give more than one spot by the thin layer technique. This is related in part to differences in fatty acid and/or fatty aldehyde composition of the lipid class. The phenomenon is most apparent when, in order to improve resolution, the sample is spotted as a small bar by applying a series of very small spots rather than one large round spot. Under these circumstances the different forms of lecithin, phosphatidyl ethanolamine, cerebrosides, and most of the other polar and ionic lipids are more readily separated and recognized as discrete spots. This can be confusing when a complex lipid mixture is examined for the first time since it is not always possible to assign each spot to a separate lipid class with certainty.

Preparation and Elution of DEAE Cellulose Columns

Because of their basic importance in the two general schemes for lipid fractionation presented here, the characteristics of the versatile DEAE columns have been explored extensively.

Nonionic lipids and zwitterion lipids are eluted from DEAE with polar nonionic solvents. We prefer mixtures of chloroform and methanol, although other solvents can be substiuted. Acidic lipids are eluted from DEAE columns with acids or bases or chloroform/methanol mixtures containing salts, acids, or bases. The most useful salt in our experience is ammonium acetate since it is quite soluble in organic

solvents and can be removed completely in many cases during evaporation of solvent (see above).

Mixtures of chloroform/methanol/salt, chloroform/acetic acid/salt and chloroform/methanol/aqueous ammonia/salt elute all acidic lipids at sufficiently high salt concentrations (0.2 M or less). When the salt content of such mixtures is gradually increased, several useful separations can be obtained. Separation into the maximum number of components is generally not as good as when appropriate mixtures of chloroform/acetic acid and glacial acetic acid are used along with solvent mixtures containing salt, and our general elution schemes therefore combine solvents containing salts with acidic and basic solvents without salt.

Since there are many possible combinations of solvents, it is apparent that DEAE offers many possibilities for special separations. We have not used mixtures of acids and alcohols in order to avoid solvent composition changes due to esterification. Similarly we have avoided the use of strong acids or bases because, even though they are effective eluting agents, they will degrade some lipids.

All preparations of DEAE tested have been found to have undesirable impurities. The types and amounts of impurities in different preparations are variable and the procedures necessary to remove the impurities will vary. The most generally useful method we have found is to wash the adsorbent with methanolic or aqueous 1 N HCl, methanol (or water), methanolic (or aqueous) 1 N KOH, and methanol (or water). Three cycles of acid and base are usually adequate. Exposure to acid and base should be as brief as possible and usually no more than three bed volumes of wash are required in each washing cycle. It is usually advisable to prepare a column from washed DEAE and run through the elution sequence for chromatography (without application of a sample) in order to insure that impurities will not be eluted when the sample is applied.

DEAE preparations differ in the number of fine particles. The finest particle sizes pack readily into columns, but with some preparations fine particles may appear along with lipid in the column effluent during some stage of the elution. With some solvents this is very pronounced. We prefer Selectacel DEAE and Selectacel DEAE type 20 (Brown Co., Berlin, N. H.) rated at 0.80–0.95 meq per g. These preparations have fewer fine particles and can be packed with ease. The Type 20 can be packed in glacial acetic acid to give a 2.5×20 cm column (using 15 g DEAE) with about 2.5 lb/in^2 pressure from a nitrogen line, but the regular grade should be packed in small portions under the same pressure and may require slight manual pressure applied with a large diameter glass plunger when a column larger than 2.5 cm i.d. is required.

Preparation of DEAE columns. DEAE columns are prepared for chromatography as follows: One hundred g of Selectacel DEAE is placed in a large, medium-porosity, sintered-glass filter over which has been placed several layers of filter paper or surgical gauze. The DEAE is then washed with 1 N aqueous HCl, water, 1 N aqueous KOH, and water, this sequence of washes constituting a cycle. After three wash cycles, the bed is washed with methanol. The bed is then air dried on the filter under mild suction from a water pump, transferred to a vacuum desiccator and thoroughly dried over KOH. A 15-g portion of this dry material (to prepare a 2.5×20 cm column) is placed in a beaker and allowed to stand overnight in glacial acetic acid. To insure thorough wetting of the ion exchange cellulose with acetic acid and a uniformly packed column, the ion exchange cellulose is pressed gently with a pestle in a mortar until it takes on a uniform appearance. The column can be packed with DEAE as a slurry in methanol, but it is more difficult to obtain a highly uniform bed with this solvent.

A 20-cm column can be packed in a 2.5 (i.d.) $\times$ 40 cm chromatography tube equipped with a Teflon stopcock. A small plug of glass wool is held in place at the bottom of the tube with a glass rod, and a slurry of DEAE in glacial acetic acid is passed into the chromatography tube. After the first addition of DEAE, the rod is withdrawn and packing is continued. Approximately five equal portions can be packed to give a satisfactory column. After each addition of DEAE, the excess acid is forced out under approximately 2.5 psi nitrogen pressure and the DEAE bed is pressed lightly with a large bore glass rod. At this stage the bed height should be 22–24 cm (with 15 g of DEAE). Two to three bed volumes of glacial acetic acid are passed through the column, and glacial acetic acid is washed out with methanol (approximately 6 bed volumes). Removal of acetic acid is assured by testing with pH paper. Methanol is washed out with chloroform, and chloroform replaced by the chloroform/methanol mixture to be used as the first eluting solvent. At this stage the bed height should be 20 ± 2 cm. Columns of larger diameter can be packed with more DEAE in proportion to the increase in volume (column diameter).

Eluting Solvents. Various elution schemes that can be recommended for particular purposes are listed below in order of increasing complexity. There are two general groups of eluting solvents. Solvents of the first group are used for elution of nonacidic lipids and solvents of the second group are used for elution of acidic lipids. Any one of the systems of the first group can be combined with solvents of the second group to make a complete elution scheme depending upon the composition of the sample and the information desired. The volume of solvent collected with different solvent mixtures varies but should be within the range of 5–10 column volumes.

Elution of Nonacidic Lipid. This category includes nonionic lipids (sterols, sterol esters, glycerides, etc.), lecithin, sphingomyelin, lysolecithin, phosphatidyl ethanolamine, and lysophosphatidyl ethanolamine as well as any new lipid classes that have the same or similar ionic groups (see work on the sea anemone below).

I. *Elution of total nonacidic lipid and water soluble nonlipid.* The column in the acetate form is prepared in methanol, the sample applied in methanol or a suitable chloroform/methanol mixture in which the sample is soluble, and the column eluted with methanol to remove all nonacidic lipids and water soluble nonlipid components. The column is then cleared of acidic lipids (see below) before it is used for another sample.

II. *Elution of total nonacidic lipid and water soluble nonlipid separately.* The column is prepared in chloroform/methanol 9/1, 7/1, or 7/3 and the sample is applied in any of these solvents. Eluting solvents are:

1) Chloroform/methanol 7/3 (total nonacidic lipids).

2) Methanol (water soluble nonlipid, lysophosphatidyl ethanolamine, and some oxidation products of phosphatidyl ethanolamine when present). The column is cleared of acidic lipids by one of the methods outlined below.

III. *Separation of pure phosphatidyl ethanolamine, remaining nonacidic lipids and water soluble nonlipid.* The column is prepared in chloroform/methanol 9/1 or 7/1 and the sample applied in either of these solvents. Eluting solvents are:

1) Chloroform/methanol 9/1 or 7/1 (elutes all nonacidic lipids except phosphatidyl ethanolamine).

2) Chloroform/methanol 7/3 (elutes pure phosphatidyl ethanolamine except when other lipid with the same ionic groups is present as in the sea anemone).

3) Methanol (elutes water soluble nonlipid, lysophosphatidyl ethanolamine, some oxidation products of phosphatidyl ethanolamine, and uncharacterized lipid present in beef liver).

The column is then cleared of acidic lipids before reuse.

Elution of Acidic Lipids. Columns are prepared and eluted as in I, II, or III above before elution of acidic lipids. Acidic lipids are then eluted as described below.

IV. *Elution of total acidic lipid with chloroform/methanol/ammonia/ammonium acetate.* Following elution with methanol to clear of nonacidic substances, elution is carried out with:

1) Chloroform/methanol/ammonia/ammonium acetate, 4/1 + 20 ml concentrated (29%) aqueous ammonia per liter + 0.05 M salt (elutes all acidic lipids).

2) Methanol wash to remove ammonia and salt.

3) Acetic acid to prepare column for reuse.

Solvent 2 can be eliminated if desired and the ammonia and salt concentration can be increased to bring about more rapid elution (decrease of elution volume), but higher salt concentrations also increase the time required for removal of salt from lipid. High salt concentrations may cause release of extraneous material from some DEAE preparations. See Figure 5 for illustration of findings with this elution sequence.

V. *Elution of total acidic lipids with chloroform/methanol/aqueous ammonia.* Following elution of nonacidic substances with methanol, elution is carried out with:

1) Chloroform/methanol/ammonia (2/1 saturated with concentrated aqueous ammonia for elution of total acidic lipids).

2) Methanol wash to remove aqueous ammonia.

3) Glacial acetic acid to prepare column for reuse.

Solvent 2 can be eliminated, but is recommended in order to avoid the formation of large amounts of ammonium acetate. The large amount of aqueous ammonia may cause the column to show channels (not visible to the naked eye) and thus the column should be repacked (in acetic acid) before it is used again. Some DEAE preparations are relatively unstable to the high concentration of aqueous ammonia used for this elution and give off extraneous material that appears in the column effluent.

VI. *Selective elution of triphosphoinositide, fatty acids, and bile acids. (Use of chloroform/glacial acetic acid 3/1).* These lipids can be eluted with chloroform/glacial acetic acid 3/1 without eluting other acidic lipids (including sulfatide, phosphatidyl inositol, cardiolipin, phosphatidic acid, ganglioside, and phosphatidyl serine). When chloroform/acetic acid 3/1 is used with brain lipid as sample (and the column has been cleared previously with methanol), only triphosphoinositide is eluted (free fatty acids, etc. not present). With this solvent and lipid from beef liver, beef heart mitochondria, or sea anemone as sample, uncharacterized lipids are eluted. Vitamin A acid and similar carboxylic acids also are eluted with chloroform/acetic acid 3/1.

Pure triphosphoinositide can be recovered from the chloroform/methanol/ammonia extract of beef brain (described above) by chromatography on a DEAE column prepared in chloroform/methanol 9/1. The first fraction eluted with chloroform/methanol 9/1 contains the nonpolar, nonionic lipid components (up to 50% of the total weight of the sample), the second fraction eluted from the column with absolute methanol contains water soluble nonlipid materials (25% or less of the total), and triphosphoinositide (about 25% of the total) is eluted with a mixture of chloroform/glacial acetic acid 3/1.

VII. *Selective elution of phosphatidic acid with chloroform/glacial acetic acid/ammonium acetate.* After elution with I, II, or III and VI above, phosphatidic acid can be eluted with chloroform/glacial acetic acid 3/1 containing 0.001 M potassium acetate or 0.005 M ammonium acetate. Other substances are also eluted with these solvent mixtures. With brain lipid as sample, uncharacterized lipid is eluted; while with beef heart mitochondrial lipid, products related to cardiolipin are obtained. It has been shown that some decomposition products of cardiolipin are eluted with this solvent. More work is required to clarify the nature of the substances related to cardiolipin that are present in brain and eluted with this mixture.

VIII. *Elution of phosphatidyl serine and ganglioside with glacial acetic acid.* Both phosphatidyl serine and ganglioside are eluted from DEAE with glacial acetic acid as well as solvents IV and V above, but are not eluted as described in I,II,III,VI, and VII above. When all nonacidic substances have been eluted first with methanol and any substances present which can be eluted with VI and VII are removed, only phosphatidyl serine and ganglioside are eluted with glacial acetic acid when cardiolipin is absent. If cardiolipin is present, it will be eluted almost quantitatively with glacial acetic acid when this solvent is preceded by solvent VII. If solvent VII is first washed out of the column with methanol (to remove salt), cardiolipin is not eluted with glacial acetic acid and only phosphatidyl serine and ganglioside are obtained.

IX. *Selective elution of cardiolipin (diphosphatidyl glycerol) with glacial acetic acid plus salt.* As noted above, phosphatidyl serine and ganglioside are eluted from DEAE with glacial acetic acid while cardiolipin is eluted with acetic acid only if solvent VII has been passed through the column first (the effective eluting solvent is acetic acid plus salt). Cardiolipin can be eluted free of other lipids if elution as in I,II, or III above is used and then the sequence VI, VIII,VII, and VIII is employed. This sequence makes use of the fact that cardiolipin is eluted with acetic

acid only when preceded by solvent VII while phosphatidyl serine and ganglioside are eluted with acetic acid without solvent VII. Glacial acetic acid plus 0.1 M salt can be substituted for solvents VII and VIII for elution of cardiolipin, but this solvent also elutes other lipids.

X. *Selective elution of sulfatide and phosphatidyl inositol with chloroform/methanol/ammonia mixtures.* As noted above (V) chloroform/methanol 2/1 saturated with concentrated aqueous ammonia elutes all acidic lipids from DEAE columns. Under the proper conditions, chloroform/methanol mixtures containing ammonia can be used for selective elutions. Chloroform/methanol 1/1, 2/1, and 4/1 each containing 10, 20, or 40 ml of concentrated ammonia per liter have been used and chloroform/methanol 4/1 containing 20 ml of concentrated ammonia per liter has been found generally useful.

The results obtained depend upon exactly how the solvent is used. Since salt increases the effectiveness of an eluting solvent, the elution scheme must take this into consideration. This is illustrated by the behavior of sulfatide of brain. Sulfatide is obtained as the magnesium salt by the method previously reported for the recovery of cerebroside plus sulfatide from a magnesium silicate column (6). When the mixture of cerebroside plus sulfatide is applied to a DEAE column, cerebroside is eluted with a chloroform/methanol mixture and sulfatide is retained. Sulfatide is eluted with chloroform/methanol 4/1 containing 20 ml of concentrated aqueous ammonia. If cerebroside is eluted with chloroform/methanol 2/1, sulfatide is eluted rapidly with chloroform/methanol/ammonia and is obtained as the magnesium salt after solvent evaporation (ammonia and ammonium acetate are removed along with solvent). If after cerebroside elution, however, the magnesium acetate formed by ion exchange of magnesium sulfatide and DEAE acetate is eluted with methanol, sulfatide is eluted less rapidly with chloroform/methanol/ammonia and is obtained as the ammonium salt after removal of solvent.

Phosphatidyl inositol is similar to sulfatide in its elution characteristics with chloroform/methanol/ammonia and this behavior can be used to obtain a mixture of phosphatidyl inositol and sulfatide from brain lipid. Nonacidic and other acidic lipids are eluted first (I,II, or III followed by VI,VII, and VIII). After removing acetic acid with a methanol wash, chloroform/methanol 4/1 containing 20 ml of concentrated aqueous ammonia per liter elutes phosphatidyl inositol and sulfatide. These lipids are eluted even more rapidly if the solvent contains ammonium acetate (0.05 M is a useful concentration).

XI. *Selective elutions with chloroform/acetic acid/ammonium acetate. (Separation of phosphatidyl serine from ganglioside and phosphatidyl inositol from sulfatide).* When chloroform/acetic acid mixtures are used with stepwise increases in concentration of ammonium acetate, several useful separations are possible. There is a wide choice of solvent compositions. The eluting capacity of these mixtures becomes greater when the amount of either acetic acid or salt is increased. Chloroform/acetic acid 1/1, 2/1, and 3/1 are all useful, but the 3/1 mixture gives the greatest degree of resolution of acidic lipids when the salt concentration is increased in steps. The upper limit of salt concentration (0.2–0.4 M) is not dependent upon solubility in the solvent, but upon the fact that solids derived from DEAE are eluted at high salt concentrations.

The elution characteristics of the complex mixture of acidic lipids of brain have been studied carefully with chloroform/acetic acid 3/1 containing from 0.001–0.4 M ammonium acetate. Useful increments are 0.001,0.005,0.010,0.025,0.050,0.10,0.20, and 0.40 M. When all of the acidic lipids of brain are present, several incompletely separated peaks are observed. Complete separations of several lipids can be obtained when less complex mixtures are applied to the column.

Phosphatidyl serine and ganglioside eluted together from DEAE with solvent VIII can be applied to another DEAE column and eluted separately using chloroform/acetic acid 3/1 containing 0.005–0.1 M ammonium acetate for elution of phosphatidyl serine and 0.2–0.4 M salt for gangliosides. With small stepwise increases in salt concentration, partial separation of the different gangliosides can be obtained.

A mixture of phosphatidyl inositol and sulfatide eluted from DEAE with solvent X above can be separated completely on DEAE by elution of phosphatidyl inositol with chloroform/acetic acid 3/1 being 0.025–0.05 M in ammonium acetate and sulfatide with the same solvent mixture containing 0.1–0.4 M salt. With small stepwise increases in salt concentration, partial separations of different types of sulfatides can be observed.

XII. *Selective elution of phosphatidyl serine with acetic acid/chloroform 4/1.* This lipid can be eluted along with ganglioside with solvent VIII. When the same general procedure is used as in VIII, but the mixture acetic acid/chloroform 4/1 is used for elution of phosphatidyl serine rather than glacial acetic acid, ganglioside is not eluted and pure phosphatidyl serine is obtained.

XIII. *Separation of phosphatidyl ethanolamine and phosphatidyl serine.* Phosphatidyl ethanolamine and phosphatidyl serine can be separated readily on DEAE since phosphatidyl ethanolamine is eluted with chloroform/methanol mixtures or methanol while phosphatidyl serine is retained and eluted with acidic or basic solvents. When a mixture of these two lipids obtained by silicic acid chromatography (8) is applied to a DEAE column, procedure I above can be used for preparation of the column and elution of phosphatidyl ethanolamine. Phosphatidyl serine can be eluted with solvents IV,V, or VIII. If whole brain lipid is applied to a DEAE column, phosphatidyl ethanolamine and phosphatidyl serine can be eluted in pure form by using III above for the preparation of the column and the elution of nonacidic lipids followed by VII, a methanol wash to remove salt, and then acetic acid/chloroform 4/1 for elution of phosphatidyl serine.

Loading of DEAE columns. The separations described above are not obtained unless the proper amount of lipid is applied to the column. The proper load for beef brain lipid is 350 mg or less for a 2.5 × 20 cm column containing 15 g of DEAE (0.8–0.9 meq/g). More or less of other mixtures may be desirable depending upon the exact composition. When a column is overloaded, acidic lipids are not retained completely and may appear in several fractions. The results of overloading are illustrated in Figure 4. The maximum load must be determined by trial and error, although 200 mg of all lipid mix-

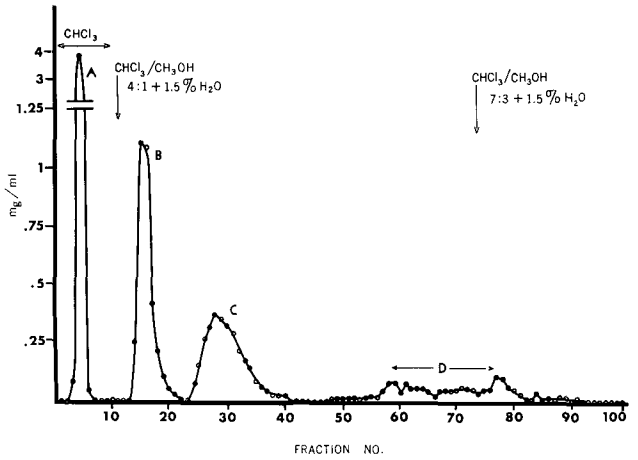


FIG. 7. Elution of cholesterol (A), cerebroside (B), lecithin (C), and sphingomyelin (D) from a silicic acid-silicate-water column (2.5 x 10 cm, i.d.). Solvent changes indicated by arrows. Fractions (10 ml) were collected at a flow rate of 3 ml/min. Sample: 152 mg of the mixture of beef brain lipids eluted from DEAE with chloroform/methanol 9/1. See text for additional comments.

tures examined in this laboratory can be applied to a 2.5 × 20 cm column without signs of overload. When more lipid is desired or required for characterization, a larger column should be used (increase of column diameter) to accept the larger amount of lipid.

Silicic Acid-Silicate-Water Columns

Silicic acid-silicate-water columns are of primary importance for the separation of fractions from DEAE columns into individual lipid classes. We have had extensive experience with three different types of columns prepared by treating silicic acid with aqueous ammonia to introduce a known amount of ammonium silicate. Such columns have the necessary characteristics for several important separations.

Silicic acid-silicate-water columns prepared by passage of a chloroform/methanol/aqueous ammonia mixture through a bed of silicic acid in a chromatography tube have been used for the separation of a mixture of phosphatidyl ethanolamine and phosphatidyl serine (8). Silicic acid-silicate-water columns for the separation of lecithin and sphingomyelin have been prepared by mixing 10 ml of concentrated aqueous ammonia with 50 g of silicic acid in enough chloroform/methanol 1/1 to make a smooth flowing slurry before transfer to a chromatography tube (6). This method gives an adsorbent of very high silicate content. The silicic acid is washed with 6 or 10 N HCl and then with water to neutrality. It is then dried before treatment with aqueous ammonia. This procedure has one disadvantage. Some silicic acid preparations give columns from which very fine particles are eluted along with lipids when the eluting solvent contains 1% or more water. This does not prevent accurate quantitation since, after evaporation of the solvent, the lipid can be extracted readily from the small amount of column material with chloroform containing 2–10% methanol.

Even traces of silicic acid or silicate can distort a solids curve of the type shown in Figure 7. For such applications an alternate procedure is useful since with it silicic acid is not eluted along with lipid. Unwashed Mallinckrodt silicic acid (50 g) in enough chloroform/methanol 1/1 to make a free flowing slurry is mixed with 7 ml of concentrated aqueous ammonia. The mixture is then passed into the chromatography tube to give a column 10 cm in height, washed with chloroform to remove both methanol and water (8), and the sample applied in chloroform. Column height should be maintained carefully since performance varies a great deal with height.

The elution of silicic acid-silicate columns can be carried out in several ways. Nonionic lipids including cholesterol, glycerides, etc., that are less polar than ceramide and cerebrosides can be eluted with chloroform (stabilized with 0.25% methanol). When chloroform/methanol mixtures without water are used for elution of the more polar lipids, some of the desired separations (including that of lecithin from sphingomyelin) are not obtained. When the correct amount of water is added to chloroform/methanol mixtures, lipids are more tightly bound to the column stationary phase, and the separation of lecithin and sphingomyelin is obtained. Polarity can be increased by increasing either the percentage of methanol or water (or both) in chloroform. We prefer to increase polarity by change in the percentage of water only. Thus, with chloroform/methanol 4/1 the water content can be varied from 0.5–3.0% with any desired series of increments for the elution of most lipids. Some samples may contain oxidized or other extraneous material or very polar lipids that can be eluted from the column with 3% water in methanol.

We have most frequently used increments of 0.5% water content beginning with chloroform/methanol 4/1 + 0.5% water for the separation of cerebrosides, lecithin, and sphingomyelin (obtained as a mixture along with cholesterol and ceramide from a DEAE column as described above). The exact findings will depend upon column height and load as well as solvent composition. Figure 7 illustrates how several lipids can be eluted with a single solvent mixture (chloroform/methanol 4/1 containing 1.5% water) but with one undesirable feature. The column was prepared by treating 50 g of silicic acid with 7 ml of concentrated aqueous ammonia as described above. The curve was prepared by weighing lipid from 1 ml aliquots of fractions on a micro-balance (8) and was not complicated by appearance of silicic acid in the fractions. Although completely separated, the cerebroside and lecithin peaks are very close together and the sphingomyelin peak is very flat. When the sequence 0.5,1.0, and 1.5% water is used, however, cerebroside and lecithin are widely separated. If a portion of the lecithin peak tails back into the sphingomyelin fraction, it can be removed by rechromatography of the sphingomyelin peak.

The relative elution order for any series of lipids can be predicted from the relative migration of the lipids with chloroform/methanol/ammonia on silicic acid paper (system 12, ref. 5). When paper chromatography indicates that two substances migrate close together, it may be wise to use increments of 0.25% water to obtain the desired separation.

General Combinations of Columns for Fractionation of Lipid Mixtures

DEAE cellulose columns are preferred for initial separation of a complex lipid mixture into fractions that can be separated conveniently into individual lipid classes by use of silicic acid, silicic acid-silicate-water, or other DEAE columns. The choice of a column for the separation of a mixture obtained from DEAE will depend upon the composition of the

sample and the information desired. The front fraction from DEAE (eluted with chloroform/methanol 7/1 or 9/1) that will contain neutral (nonionic) lipids, cerebroside, lecithin, sphingomyelin, and lysolecithin if these are present in the mixture can be handled in several ways. If only the separation of individual neutral lipids is desired and the amount of cerebroside and individual phospholipids are not required, a silicic acid column can be used. The silicic acid is washed, heat activated, and deoxygenated as described by Rouser et al. (8), prepared in hexane, and eluted essentially according to Barron and Hanahan (14). Fractions containing hydrocarbons, sterol esters, triglycerides, sterols, and lower glycerides are eluted with mixtures of ether and hexane or benzene and hexane of increasing polarity. The cerebrosides and phospholipids can then be eluted together with methanol. The elution of neutral lipids is not complicated by the presence of fatty acids or bile acids since these are removed on DEAE prior to application to silicic acid.

When cerebrosides and phospholipids of the chloroform/methanol 9/1 (or 7/1) fraction from DEAE are to be separated and the less polar neutral lipids determined as a group, a silicic acid-silicate-water column is chosen and eluted as described previously (6). Under these conditions lecithin and sphingomyelin can be separated, although the two lipids can not be separated completely on silicic acid columns. If all neutral lipids, cerebroside, and phospholipids are to be separated into individual classes, the mixture from DEAE can be applied to a silicic acid-silicate column first and eluted to give neutral lipids as a mixture, and cerebrosides, lecithin, sphingomyelin, and lysolecithin as separate fractions. The neutral lipids are then separated on a silicic acid column.

Other fractions from DEAE can be separated by silicic acid, silicic acid-silicate, or DEAE columns. Phosphatidyl ethanolamine and ceramide aminoethylphosphonate can be separated completely on silicic acid columns as described below. A mixture of phosphatidyl serine and ganglioside can be separated on silicic acid columns by elution with chloroform/methanol 4/1 for phosphatidyl serine (8) and then methanol for gangliosides. This separation can also be made on DEAE as described above. Silicic acid-silicate columns are suitable for the separation of some acidic lipids eluted from DEAE columns with chloroform/acetic acid/ammonium acetate. The separation of a mixture of phosphatidyl inositol and sulfatide can be accomplished best on DEAE by elution with chloroform/acetic acid 3/1 with increasing amounts of ammonium acetate (see above).

Column combinations including magnesium silicate–DEAE and cellulose–DEAE are of more limited use. The elution of magnesium silicate columns to give cholesterol, ceramide, and cerebroside plus sulfatide has been described (6). The mixture of cerebrosides and sulfatides can be separated on DEAE (6). The separation is obtained readily since cerebroside is not retained to any appreciable extent by DEAE. Sulfatide is eluted from magnesium silicate columns as the magnesium salt and can be obtained from DEAE columns either as the magnesium or ammonium salt depending upon the elution scheme employed as discussed above.

If small amounts of other lipids are eluted along with cerebroside and sulfatide from magnesium silicate columns through improper performance of the column, pure sulfatide can still be obtained from DEAE by elution of contaminating substances with other solvents before elution of sulfatide.

Cellulose columns can be used to separate total brain lipid into water soluble nonlipid, gangliosides, and other lipids as noted before (6). These columns are difficult to reproduce because the chloroform/methanol/water mixtures required for the separation may lead to the development of invisible channels in the column and fraction overlap may be observed. We have not developed a procedure where this effect can be controlled, and thus cellulose columns cannot be recommended for routine use.

Infrared Examination of Lipids and Lipid Hydrolysis Products

General observations. Infrared spectra of lipids in the 2–15μ region obtained from potassium bromide pellets or films over silver chloride are valuable as an aid in confirmation of identifications made on the basis of chromatographic data, and for recognition of chromatographic adsorbents in column fractions (6). The complexity of the spectra of lipids and their similarities in many respects limits the usefulness of the spectra for conclusive, independent evidence pertaining to the structure of a given lipid.

Generally, infrared spectra must be interpreted in the light of other data. The fact that absorption due to one functional group may be shifted in position owing to the influence of other groups may make positive assignment of any particular band to a particular functional group difficult. This is particularly true when a limited amount of information on lipids is available and interpretations must be made by analogy to other organic compounds where possibilities for error are great. More information on the infrared absorption of well characterized lipids and closely related compounds is required for more complete interpretations. The value of infrared is well illustrated by the work reported below on the lipids of the sea anemone.

The spectra of a number of pure lipids isolated by column chromatography have been prepared using a Beckman IR-4 double beam infrared spectrophotometer (sodium chloride optics) equipped with a beam condenser and micro pellet sample holder. The characteristic spectra of the zwitterion lipids lecithin, phosphatidyl ethanolamine, and sphingomyelin as well as the recently isolated ceramide aminoethylphosphonate are shown in Figures 8,9,10, and 39. Synthetic lipids, particularly the phosphatidyl ethanolamines, prepared from saturated fatty acids are not generally suitable for comparison by the film or potassium bromide pellet techniques with the same lipids isolated from tissues. The lipids synthesized with saturated fatty acids give distinctly different spectra (Fig. 11). The saturated compounds are solids that have absorption characteristics that may be related to different crystalline forms not obtained with the unsaturated, natural forms. When the natural phosphatidyl ethanolamines are hydrogenated, the spectra resemble the saturated synthetic products, and when partially hydrogenated an intermediate spectrum is obtained (Fig. 12). The spectra obtained in this laboratory for the saturated phosphatidyl ethanolamines are different in several respects from spectra presented by others (15,16) with the pellet technique. The spectra obtained from

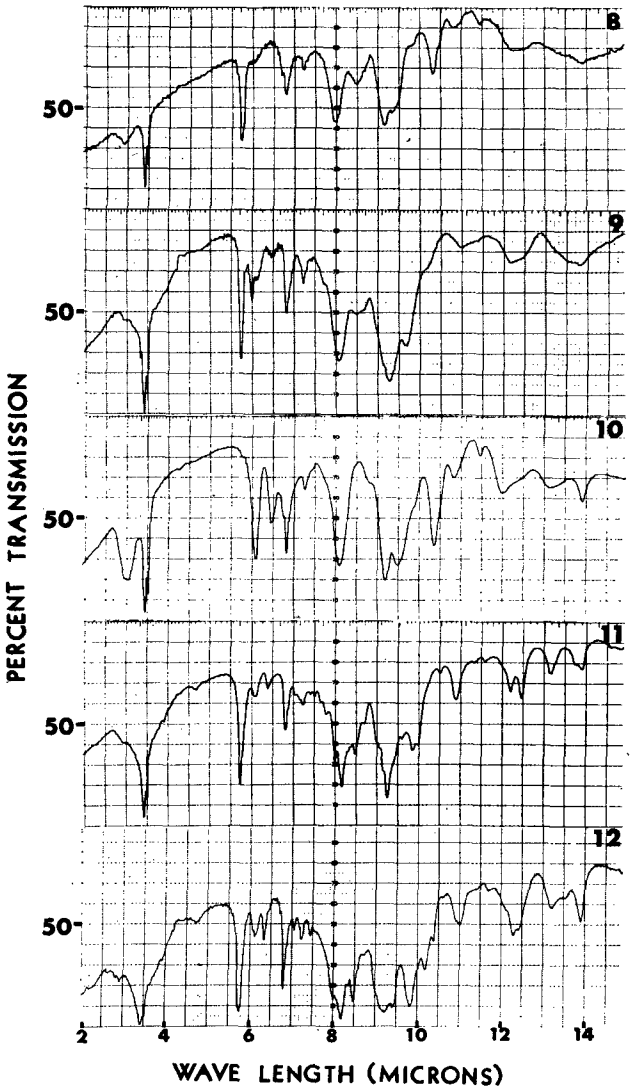


FIG. 8. Infrared spectrum of beef brain lecithin isolated from a silicic acid-silicate-water column and prepared as a 0.19 mm thick KBr pellet containing 1.22% lipid. See text for comments.

FIG. 9. Infrared spectrum of beef brain phosphatidyl ethanolamine isolated from a DEAE cellulose column (see text for details). Sample prepared as a KBr pellet 0.16 mm thick containing 2.34% lipid. See text for comments.

FIG. 10. Infrared spectrum of brain sphingomyelin isolated from a silicic acid-silicate-water column and prepared as a KBr pellet 0.310 mm thick containing 1.36% lipid. See text for comments.

FIG. 11. Infrared spectrum of synthetic dimyristoylphosphatidyl ethanolamine (gift from Erich Baer) prepared as a 0.158 mm thick KBr pellet containing 1.71% lipid. See text for comments.

FIG. 12. Infrared spectrum of partially hydrogenated phosphatidyl ethanolamine. Phosphatidyl ethanolamine of soybean was isolated from a DEAE column, hydrogenated over a palladium catalyst, and examined as a KBr pellet. See text for comments.

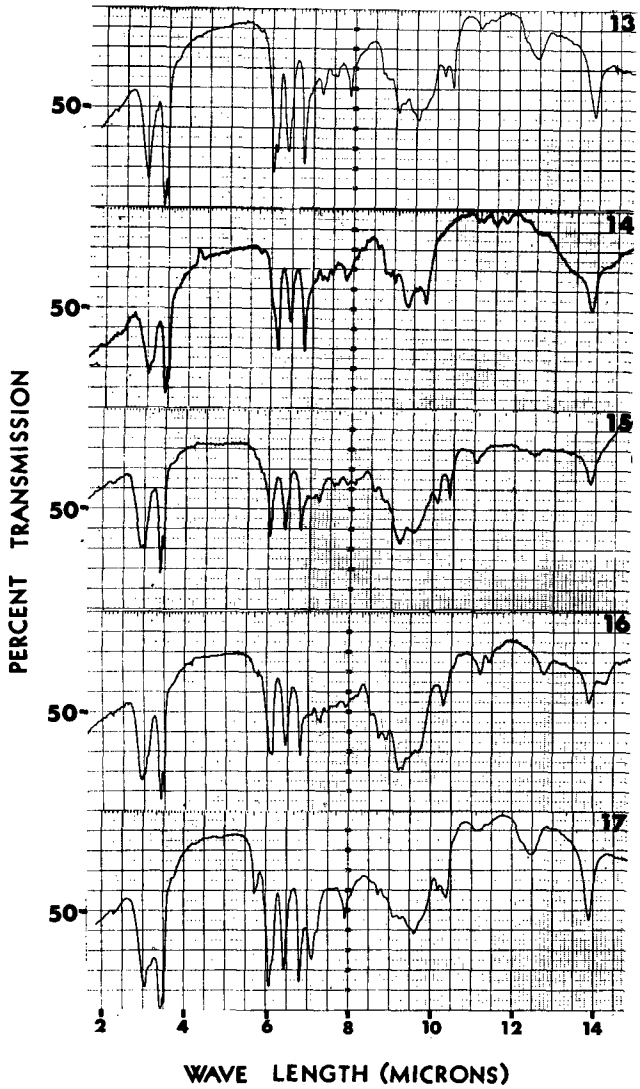


FIG. 13. Infrared spectrum of ceramide prepared by enzymatic degradation of sphingomyelin (gift from Michael Sribney) prepared as a 0.316 mm thick KBr pellet containing 1.47% lipid. See text for comments.

FIG. 14. Infrared spectrum of yeast cerebrin. The sample was eluted from a Florisil column with chloroform/methanol 4/1 and examined as a KBr pellet. See text for comments.

FIG. 15. Infrared spectrum of cerebroside isolated from the spleen lipids of a patient with Gaucher's disease. The sample was eluted from a Florisil column and examined as a KBr pellet. See text for comments.

FIG. 16. Infrared spectrum of beef brain cerebroside isolated by column chromatography on magnesium silicate and DEAE cellulose. The sample was prepared as a 0.170 mm thick KBr pellet containing 3.47% lipid. See text for comments.

FIG. 17. Infrared spectrum of ceramide after heating with 0.05 N HCl for 6 hrs in a sealed tube at 100C. The sample was prepared as a 0.138 mm thick KBr pellet containing 4.75% lipid. Note the strong increase of the ester absorption band at 5.72 μ brought about by acid (compare with Figure 13 before acid treatment). See text for comments.

phosphatidyl ethanolamine of beef brain, soybean, beef heart mitochondria, and the sea anemone are very similar to each other and to the spectrum reported by Baer and Buchnea (17) for dioleylphosphatidyl ethanolamine in KBr. The natural phosphatidyl ethanolamines isolated from different sources give spectra that are very similar despite the fact that they differ in fatty acid composition and that some contain almost no plasmalogen form (soybean) while others occur predominantly as the plasmalogen form (brain).

The fact that differences in chain length, unsaturation, and the presence of the α,β-unsaturated ether link of the plasmalogen form do not change the spectra of naturally occurring phosphatidyl ethanolamines appreciably facilitates infrared comparisons of this lipid class from different sources. The differences in spectral characteristics after hydrogenation are useful in comparisons. Natural products can be hydrogenated and compared to other fully saturated lipids as an aid in characterization.

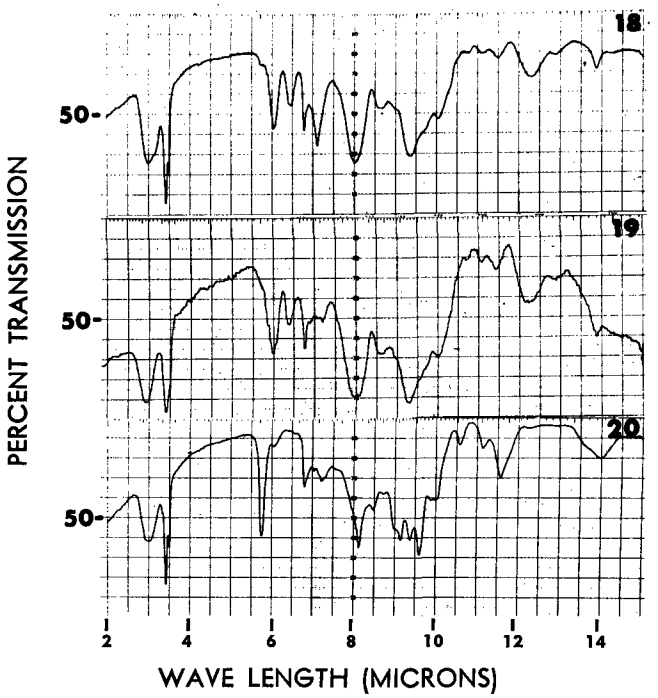


FIG. 18. Infrared spectrum of beef brain sulfatide isolated by a combination of magnesium silicate and DEAE cellulose column chromatography (see text for details). Sulfatide was evaporated with an excess of concentrated aqueous ammonia prior to examination and prepared as a 0.175 mm thick KBr pellet containing 1.81% lipid. Note the relatively strong sharp band at 7.10 μ and the shouder at 3.15 μ characteristic of the ammonium salts of acidic lipids not seen in a similar preparation isolated from a silicic acid column (compare with Fig. 19). Note also the small absorption at 5.72 μ indicating a trace of glycerol lipid. See text for comments.

FIG. 19. Infrared spectrum of beef brain sulfatide isolated as described for the preparation in Figure 18 and then passed through a silicic acid column. Note the absence of the peaks at 7.10 and 3.15 μ characteristic of the ammonium salt (seen in Fig. 18) and the absence of the ester absorption at 5.72 μ after removal of a trace of glycerol phosphatide present in the sample in Figure 18. See text for further comments.

FIG. 20. Infrared spectrum of the sodium salt of phosphatidyl inositol isolated from wheat germ (gift from Dr. M. J. Morelee). Sample prepared as a 0.148 mm thick KBr pellet containing 1.42% lipid. Compare with Figures 21 and 22 prepared from beef brain phosphatidyl inositol. See text for further comments.

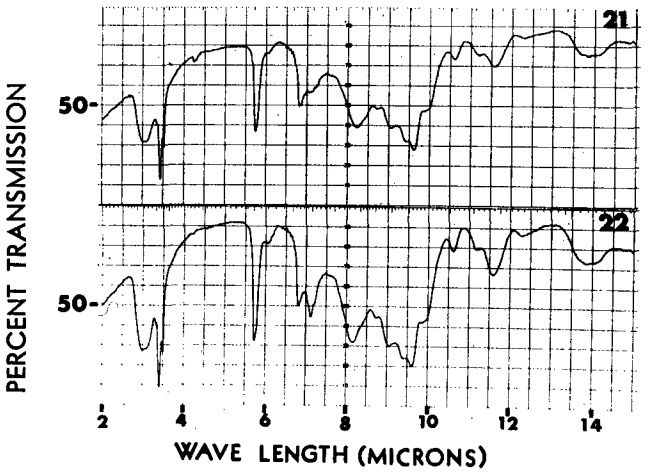


FIG. 21. Infrared spectrum of beef brain phosphatidyl inositol by a combination of DEAE cellulose and silicic acid column chromatography. The sample was eluted from silicic acid with chloroform/methanol 3/2 and then examined as a 0.121 mm thick KBr pellet containing 1.84% lipid. Note the absence of a distinct band at 7.1 μ characteristic of the ammonium salt of phosphatidyl inositol. The sample was applied to the column as the ammonium salt, but was partially converted to the acid form by silicic acid. Compare with Figure 22 prepared from the same sample after evaporation with chloroform/methanol/aqueous ammonia to obtain the ammonium salt. See text for further comments.

FIG. 22. Infrared spectrum of brain phosphatidyl inositol prepared from the same sample shown in Figure 21 after evaporation with chloroform/methanol/aqueous ammonia for conversion to the ammonium salt. It was prepared as a 0.159 mm thick KBr pellet containing 1.89% lipid. Note the strong bands at 7.10 and 3.15 μ that are characteristic of the ammonium salts of acidic lipids. See text for further comments.

Infrared examination is useful in distinguishing the glycerol lipids from sphingolipids. A comparison of lecithin, phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidyl serine (Figs. 8,9,20,25) with sphingomyelin, ceramide, yeast cerebrin (also a ceramide), cerebroside, and ceramide aminoethylphosphonate (Figs. 10,13,14,15,39) clearly shows the presence of a strong band at 5.70–5.75 μ (related to the absorption of the ester carbonyl group of the glycerolphosphatides) that is absent from the sphingolipids. Absorption at ca. 6.1 μ (amide I band) is characteristic of sphingolipids, although all glycerophosphatides examined have some absorption in this region, and phosphatidyl serine (Fig. 25) shows a relatively strong band at 6.1 μ. The amide II band near 6.4 μ is fairly strong in the sphingolipids. Absorption is not seen around 6.4 μ with pure lecithin or phosphatidyl inositol, but some absorption is seen in this region in the spectrum of phosphatidyl ethanolamine and phosphatidyl serine. Since this band is related to –NH absorption, its presence in the spectrum of lipids with amino groups as well as amides and its absence from the spectrum of lecithin and phosphatidyl inositol is readily understood.

The 5.70–5.75 μ absorption of beef brain cerebroside. Since sphingolipids do not have absorption in the ester carbonyl region (5.70–5.75 μ), we were surprised to find that column chromatography frequently gave preparations with some ester absorption. In the case of sphingomyelin this was related to a small contamination with lecithin. To remove the last traces of this contaminant it is sometimes necessary to rechromatograph the sphingomyelin fraction from a silicic acid-silicate-water column to free sphingomyelin of the last traces of lecithin. The small contamination may be missed by paper chromatography when it is easily detected by infrared examination. Beef brain cerebroside isolated by column chromatography has invariably shown the presence of a small ester band. This is also seen occasionally in spectra of sulfatide (Fig. 18). The small 5.75 μ absorption of some sulfatide preparations was readily removed by rechromatography. It is evidently related to an occasional slight contamination (ca. 1%) of sulfatide with an uncharacterized acidic phospholipid that gives a positive ester test (hydroxamic acid). In the cerebroside fractions the absence of glycerol containing lipids known to occur in animal tissues was demonstrated by column, paper, and thin layer chromatography. These preparations did not contain phosphorus and a clear demonstration of glycerol was not possible. The ester hydroxamic acid test, however, was positive.

The possibility that the ester absorption of cerebroside preparations from beef brain might be related to changes produced by autoxidation or to an N to O acyl migration during column chromatography was considered. The band at about 5.75 μ was still

observed even when all steps were carefully conducted under nitrogen indicating that the absorption was not an artifact of oxidative change related to column chromatography. The possibility of a significant N to O acyl migration to produce an ester group during column chromatography was then excluded by passing sphingolipids without ester absorption through the same column chromatographic procedures used for the isolation of cerebrosides. No significant ester absorption was introduced and cerebroside was ninhydrin negative. A further indication that the N to O migration was unlikely was obtained by treatment of sphingolipid preparations with aqueous acid. When ceramide, cerebroside, and sphingomyelin were heated individually in sealed tubes with 0.05 N HCl for 2–20 hr, only ceramide showed a large increase in ester absorption after 6 hr (Fig. 17). The other lipids suffered extensive hydrolysis without appreciable increase in ester absorption. It thus appears that more vigorous conditions than those encountered in chromatography are required to produce an N to O acyl migration.

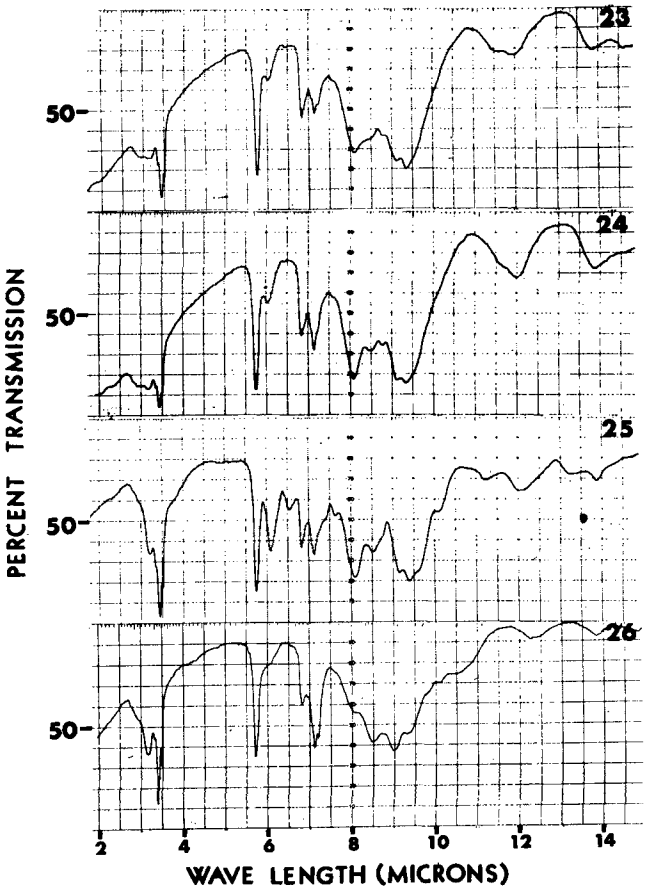


FIG. 23. Infrared spectrum of cardiolipin isolated from beef heart mitochondria by DEAE cellulose column chromatography as described in the text. The sample was evaporated directly from a mixture of chloroform/acetic acid containing ammonium acetate and examined as a 0.310 mm thick KBr pellet containing 1.22% lipid. The preparation was partially in the free acid from and after evaporation with concentrated aqueous ammonia some of the absorption characteristics were changed (see Fig. 24). See text for further comments.

FIG. 24. Infrared spectrum of the same preparation of cardiolipin shown in Figure 23 after addition of excess concentrated aqueous ammonia to convert to the ammonium form. The increased strength of the absorption at 7.12 and 3.15 μ characteristic of the ammonium salt is evident (compare with Fig. 23). See text for further comments.

FIG. 25. The infrared spectrum of beef brain phosphatidyl serine isolated from a DEAE cellulose column (eluted with chloroform/acetic acid 3/1 containing ammonium acetate as described in the text). The sample was in the ammonium form and bands at 7.12 and 3.20 μ are strong. It was prepared as a 0.15 mm thick KBr pellet containing 1.84% lipid. See text for additional comments.

FIG. 26. Infrared spectrum of beef brain phosphatidic acid isolated first from DEAE cellulose and then from a silicic acid-silicate-water column (see text for details). Sample prepared as a 0.162 mm thick KBr pellet containing 1.29% lipid. The preparation was predominantly in the ammonium form and the 7.10 and 3.15 μ absorptions characteristic of the ammonium salts of the acidic lipids are relatively strong. See text for further comments.

The data clearly indicated that the ester absorption was a characteristic of the preparations and not an artifact. Since keto acids have been shown to have absorption in this region, it was thought that the absorption might arise from a keto acid in the sphingolipid molecule. This possibility was checked by passage of brain cerebroside treated with 2,4-dinitrophenylhydrazine hydrochloride through a silicic acid column. After elution of excess reagent with chloroform, cerebroside was eluted with chloroform/methanol 9/1. No indication of the formation of a derivative of cerebroside was obtained but a colored product, possibly a hydrazide, was formed.

Repeated attempts to separate another component from cerebroside were unsuccessful and the fully acetylated cerebroside (pyridine-acetic anhydride) showed only one spot after paper chromatography. Since only galactose was detected by paper chromatography after acid hydrolysis (3 N HCl, 1.5 hr, 100C, sealed tube), it was concluded that the brain cerebroside was contaminated with a glycerol lipid containing galactose. Both mono and digalactosylglycerides have been isolated from plants and the products of mild alkaline hydrolysis have been placed on chromatograms. After deacylation of our cerebroside preparation with alkali we obtained a spot having the correct paper chromatographic migration for galactosylglycerol. In keeping with the findings of Steim and Benson (18) this suggests the presence of monogalactosyldiglyceride.

Infrared absorption of acidic lipids. Acidic lipids present a special problem since the spectra obtained will depend upon the form examined. The free acid and the salt forms give different spectra. Furthermore, ammonium salts have absorption not seen in the spectra of the sodium or potassium salts.

The spectrum of the sodium salt of phosphatidyl inositol from wheat germ is shown in Figure 20. The spectrum from brain phosphatidyl inositol applied to a silicic acid column as the ammonium salt and eluted with chloroform/methanol 3/2 is shown in Figure 21 while Figure 22 shows the same preparation after evaporation with excess ammonia to convert back completely to the ammonium form. It is evident that absorption in the 3.15 and 7.1μ regions is strong for the ammonium salt. These differences can be seen when sulfatide as the ammonium salt (Fig. 18) is compared to sulfatide isolated from a silicic acid column (Fig. 19). This is illustrated further by the spectra of the ammonium salts of cardiolipin (Figs. 23,24), phosphatidyl serine (Fig. 25), and phosphatidic acid (Fig. 26). Although there are differences in other regions of the spectra, these are generally less pronounced than those associated with absorption of the ammonium ion.

The spectra of acidic lipids prepared in this laboratory have been obtained largely from ammonium and magnesium salts since these are the forms usually eluted from DEAE and magnesium or ammonium silicate columns. If eluted in the less stable acid form,

acidic lipids can be conveniently converted to the ammonium salts by evaporation with excess ammonia. (Compare Figs. 21 and 22 showing conversion to the ammonium salt.) The ammonium or magnesium salts can be converted to sodium or potassium salts by passage through a short bed of the sodium or potassium forms of moisture free phosphocellulose.

General interpretations of spectral characteristics are rather uncertain. An illustration of a typical difficulty is afforded by absorption in the *trans* double bond region (10.3μ). Sphingolipids absorb in this area when the *trans* double bond of sphingosine is present, and may not absorb when it is absent as with yeast cerebrin (Fig. 14). Lecithin without a *trans* double bond absorbs at 10.3μ and some cerebroside preparations absorb at 10.2 and 10.4μ rather than 10.3μ (Fig. 15). The disappearance of the 10.3 (or 10.2 and 10.4) absorption after hydrogenation can be used to demonstrate *trans* double bond absorption.

Infrared spectroscopy of hydrolysis products of lipids. Infrared spectroscopy is a valuable supplement to chromatographic techniques for characterization of hydrolysis products of lipids. Infrared examinations can be carried out in several ways. Different hydrolytic conditions can be employed ranging from mild acid or alkali to strong acid or alkali for varying times at different temperatures. Under any given set of hydrolytic conditions it is possible to examine all the hydrolysis products together (i.e. the fatty acids, etc. along with water soluble components) or various separations can be carried out prior to infrared study. Separation of hydrolysates into lipid and water soluble fractions by solvent partition prior to infrared examination is a convenient, simple, and rapid procedure and is used as an aid in characterization in this laboratory.

The essential steps in the method are: 1) hydrolysis of the sample and, if available, an authentic lipid for comparison; 2) separation of the hydrolysis products into water soluble and organic solvent soluble fractions; 3) determination of the weight of water soluble and organic solvent soluble hydrolysis products (when salt is present, the water soluble portion cannot be weighed without further purification); 4) preparation of appropriate solutions of the two fractions for chromatography and infrared examination; and 5) preparation of a KBr pellet from weighed amounts of sample and KBr and preparation of the spectrum.

Control samples for the water soluble and organic solvent soluble portions of hydrolysates can be prepared from known amounts of authentic substances (glycerophosphate, choline, fatty acids, etc.) rather than from samples of authentic lipids obtained by synthesis or isolation. When carefully weighed amounts of samples and KBr are mixed and pressed into pellets of the same size, any deviations from the control sample have both qualitative and quantitative significance.

The problems encountered in the examination of hydrolysates of phosphatidyl ethanolamine can be used to illustrate the method. Both the diacyl and plasmalogen forms occur in brain and the hexane soluble products will include a mixture of fatty acids and fatty aldehydes. Under the hydrolytic conditions described by Rouser et al. (8) both plasmalogen and diacyl forms give only glycerophosphate and ethanolamine as the water soluble products. Since no trace of ethanolamine-O-phosphate is formed, the infrared spectrum of the water soluble hydrolysis products should be that of an equimolar mixture of glycerophosphate and ethanolamine provided the same ionic forms are examined in both control and experimental runs. This can be achieved by running an authentic sample of phosphatidyl ethanolamine along with the sample under investigation, or more simply by mixing glycerophosphate and ethanolamine in equimolar amounts and subjecting the mixture to the same conditions as the sample. When the KBr pellet technique is used with sodium chloride optics (as in our own studies), the presence of sodium or potassium chlorides in the samples is of no significance for infrared examination. The sodium or potassium salt of glycerophosphate and ethanolamine rather than ethanolamine hydrochloride can be used to prepare the authentic sample used for comparison with a lipid hydrolysate provided the authentic mixture is treated with HCl prior to infrared examination. If this is not done the standards, due to differences in ionic form, will not correspond exactly to the same substances obtained from acid hydrolysis of lipids. Differences, attributable to variations in –NH absorption, are very marked in the 3.15 and 7.1 μ regions of the spectrum.

After mild alkaline hydrolysis, glycerylphosphoryl ethanolamine is formed from the diacyl form of phosphatidyl ethanolamine, but only one fatty acid is released from the plasmalogen form to give a lyso compound. Acid and alkaline hydrolysis of lecithin and phosphatidyl serine show an analogous behavior. Acid hydrolysis of phosphatidyl inositol releases glycerol, inositol, inositol phosphates, and glycerophosphates, thus the most appropriate standard is an authentic phosphatidyl inositol sample hydrolyzed in the same manner. This would be the best general procedure also with cardiolipin and similar substances. Acid hydrolysis of sphingomyelin will give phosphorylchlorine, sphingosine, and fatty acids as hydrolysis products while cerebroside will give galactose (and/or glucose depending upon the source), sphingosine, and fatty acids. An equimolar mixture of sphingosine and fatty acids gives a characteristic spectrum as do phosphorylcholine and the hexoses.

Lipid Composition of Beef Liver

The findings with beef liver lipids illustrate the value of DEAE column chromatography combined with paper and thin layer chromatography (scheme one) for recognition of the variety of lipid classes that may be present in an organ and not be detected when paper and thin layer chromatography alone are used. After DEAE column chromatography, many more lipid classes can be recognized. Several new lipid classes have been detected in liver and the presence and approximate amounts of phosphatidyl inositol and cardiolipin have been confirmed, while both phosphatidic acid and lysolecithin that have been reported to occur in liver were not detected.

Extraction of beef liver. Fresh beef liver was frozen less than 30 min from the time of death. A 100 g portion was later extracted with 2 liters of chloroform/methanol 2/1, the solvent removed using the rotary evaporator, and the lipid dried over KOH.

DEAE column chromatography. Columns 4.5 × 20 cm were prepared as described above using 45 g of DEAE. Samples (750–800 mg) of crude lipid were applied to the columns in chloroform/methanol

TABLE II
Lipid Composition of Beef Liver

Solvent	Substances	% Total Lipid
(1) C/M 9/1	Nonionic lipids. lecithin, sphingomyelin	45.7
(2) C/M 7/3 and CH_3OH	Phosphatidyl ethanolamine, uncharacterized lipid, and water soluble nonlipids	34.8
(3) C/HAc 3/1	Uncharacterized	8.7
(4) C/HAc 3/1 + 0.05 M NH_4Ac	Cardiolipin, phosphatidyl serine, phosphatidyl inositol, and several minor uncharacterized lipids*	8.8
(5) HAc	Uncharacterized	1.0
(6) C/M 4/1 + 20 ml/liter conc. aq. ammonia + 0.01 M NH_4Ac	Uncharacterized	0.6
	Total acidic lipid=19.1% Total nonacidic lipid=80.5%	99.6%

* Approximate percentages of components obtained by silicic acid chromatography are: cardiolipin (plus some related products), 1.4%; phosphatidyl serine, 0.6%; "phosphatidyl inositol" (2 closely related substances), 5.2%.
C = chloroform, M = methanol, HAc = acetic acid, NH_4Ac = ammonium acetate.

9/1. Three DEAE columns were run. The elution sequence was chloroform/methanol 9/1, chloroform/methanol 7/3, methanol, chloroform/acetic acid 3/1, chloroform/acetic acid 3/1 plus 0.05 or 0.10 M ammonium acetate, glacial acetic acid, methanol wash, and chloroform/methanol 4/1 containing 20 ml of concentrated aqueous ammonia per liter and 0.01 M ammonium acetate.

The percentage of lipid eluted with the various solvents is shown in Table II. The chloroform/methanol 7/3 and methanol eluates are shown as one fraction since phosphatidyl ethanolamine was not separated completely from uncharacterized lipid eluted with both solvents. Water soluble nonlipid was present also in the methanol eluate. Several uncharacterized lipid components in the methanol eluate can be visualized by thin layer chromatography (Figs. 27,28). The substances are evidently not acidic since they are eluted with a nonionic solvent (methanol). The lipid is quite soluble in methanol and relatively insoluble in chloroform. This accounts for the failure of chloroform/methanol 9/1 and 7/3 to elute the lipid rapidly from DEAE. The major component migrates just behind lecithin on paper chromatograms and just ahead of lecithin on thin layer chromatograms. This new lipid is a very polar methanol soluble nonacidic substance. The complete characterization of this material should be interesting since it represents several per cent of the total liver lipid and does not appear to have been encountered in previous investigations.

The chloroform/acetic acid 3/1 eluate is surprisingly large (8.7% of the total lipid) and several uncharacterized substances can be seen by thin layer chromatography (Fig. 27). Fatty acids, bile acids, vitamin A acid, etc. are eluted with this solvent. Some bile acid and free fatty acid may be present in this fraction, but complete separation into individual components on other columns will be necessary for complete characterization.

Paper and TLC indicated that the chloroform/acetic acid/ammonium acetate fraction probably contained cardiolipin and phosphatidyl inositol and the mixture was fractionated further on a silicic acid column. Despite some fraction overlap, approximate values for some of the components were obtained and are shown below in Table II. We failed to detect material with the properties of phosphatidic acid in disagreement with the reports of Hübscher (19,20) although a trace (0.1% or less of the total lipid) might have escaped detection. Our results agree with those of Macfarlane (21) who found cardiolipin and related products and suggested that these substances may have been the products isolated by Hübscher and Clark (19).

The phosphatidyl inositol fraction isolated from the silicic acid column was eluted largely with chloroform/methanol 3/2 as expected from the work of Hanahan et al. (22), but some was found in the chloroform/methanol 4/1 eluate as well. Phosphatidyl inositol from liver had the same column chromatographic characteristics as phosphatidyl inositol from soybean, wheat germ, and brain. All of the preparations were similar by paper and TLC and by infrared spectroscopic examination, although the preparation from liver showed two spots after paper and thin layer chromatography. Inositol, inositol phosphate, glycerophosphate, and glycerol were detected in acid hydrolysates from the preparations.

Discussion of beef liver results. The present findings are to be compared with the results obtained by others using silicic acid column chromatography or thin layer chromatography. In these comparisons the neutral lipids appearing at the front on DEAE columns will not be considered, since these were not studied further. Hanahan et al. (22) fractionated beef liver lipids on silicic acid columns and recognized the presence of phosphatidyl ethanolamine, phosphatidyl serine, phosphoinositide, lecithin, and sphingomyelin in column eluates. Neither paper nor TLC was used. Since these investigators washed their total lipid extracts with water and then precipitated and washed the lipid with acetone, some differences in composition from the crude chloroform/methanol extracts examined in this laboratory can be expected.

Getz et al. (23) studied the lipid composition of rat liver by silicic acid chromatography in much the same manner as Hanahan et al. (22). Polar lipid fractions were reported to contain phosphatidyl ethanolamine, phosphatidyl serine, inositol phosphatide, lecithin, and sphingomyelin. They noted also, as did Hanahan et al., that an early fraction might contain cardiolipin (polyglycerolphosphatide).

Nelson (24) studied the lipids of mouse liver by silicic acid column chromatography and examined column fractions by TLC with chloroform/methanol 2/1 plus 2% water as solvent and by infrared spectroscopy of chloroform or carbon tetrachloride solutions. Aside from neutral lipids, he noted the presence of phosphatidyl ethanolamine and serine, monophosphoinositide, lecithin, lysolecithin and sphingomyelin.

Skipski et al. (25) used one dimensional TLC with "neutral" and "basic" adsorbents developed with mixtures of chloroform/methanol/acetic acid/water. Using a general detection reagent they were able to observe spots attributed to phosphatidyl ethanolamine, phosphatidyl serine, lecithin, sphingomyelin, and lysolecithin. Skidmore and Entenman (26) used two dimensional TLC with two different chloroform/methanol/ammonia mixtures as solvents to study rat liver lipids. With a general detection reagent (iodine vapor) they observed spots in the correct positions for phosphatidic acid, phosphatidyl ethanolamine and serine, phosphatidyl inositol, lecithin, sphingomyelin and lysolecithin.

It is clear that previous investigators did not encounter the very large number of lipid components

observed in the present studies. Previous investigators observed 5–7 components and occasionally noted the possible presence of cardiolipin. Our studies disclosed some 30 or more polar lipids in beef liver when all definitely different spots on thin layer chromatograms prepared from DEAE column fractions are counted. Some of the lipids appear to be new classes since they are eluted from DEAE columns with solvents that differentiate them from the known lipids.

There are several reasons for such marked differences in lipid composition depending upon the methods used. First, the use of untreated chloroform/methanol 2/1 extracts to avoid possible loss of lipid by water washing or acetone precipitation is important. Second, the overlap of fractions on silicic acid columns and spots on thin layer plates (coupled with the relatively small amount of some of the components) can lead to a failure to recognize all of the components. We observed only a small number of spots when either one or two dimensional TLC was carried out on liver lipid extracts in keeping with this conclusion (Figs. 27,28).

The major advantage of the present procedure over previous methods lies in the high resolving power of the DEAE columns. Another distinct advantage of the present procedure is the use of both paper and TLC after column chromatography. This advantage is well illustrated by findings for lysolecithin. With TLC we observed a spot in approximately the correct position for lysolecithin in keeping with the reports of Skipski et al. (25) and Skidmore and Entenman (26). By paper chromatography in systems 11,12,15, and 16 (5) the spot was clearly not lysolecithin. No trace of lysolecithin could be found. We have not been able to obtain a satisfactory spot for phosphatidic acid by TLC, but paper chromatography gives a good, well-defined spot. Paper chromatography is also more useful than TLC for distinguishing phosphatidyl serine from other lipids while TLC as described above is very useful for recognition of cardiolipin in a lipid mixture.

The findings with regard to cardiolipin and phosphatidyl inositol deserve further comment. Faure et al. (27) isolated cardiolipin (polyglycerophosphatide) from ox liver and showed it to be similar to the cardiolipin isolated from beef heart. Macfarlane (21) also demonstrated the presence of cardiolipin in ox liver and the percentage found by her (1–2% of the total phospholipid) is in agreement with the value of 1.4% of the total lipid found in the present studies.

Hanahan et al. (22) and Faure et al. (27) reported the isolation of phosphatidyl inositol from beef liver and its presence in liver was confirmed in the present studies. The chromatographic approach involving both DEAE cellulose and silicic acid column chromatography disclosed, however, a closely related lipid as a contaminant in the phosphatidyl inositol fraction.

Lipid Composition of the Sea Anemone

Our interest in the lipid composition of the sea anemone was first aroused by the work of Bergmann and Landowne (28) who reported that *Anthopleura elegantissima* contained sphingomyelin and a small amount of choline plasmalogen, but no lecithin, phosphatidyl ethanolamine, or other common polar lipids. We examined a lipid extract of this species by paper chromatography: no trace of sphingomyelin was detected but a number of neutral lipids (including sterols and glycerides), and lecithin, phosphatidyl ethanolamine, and phosphatidyl serine along with other polar lipids were detected. These results were thus exactly the opposite of those reported by Bergmann and Landowne. Interest in this problem was again aroused when Kittredge et al. (29) isolated free 2-amineothylophosphonic acid from *Anthopleaura elegantissima* and reported that alkali released 2-aminoethylphosphonic acid from lipid extracts. These findings suggested that 2-aminoethylphosphonic acid with a carbon-phosphorus bond was incorporated into a phospholipid and the problem of sea anemone lipids was reinvestigated.

The studies of the lipids of the sea anemone illus-

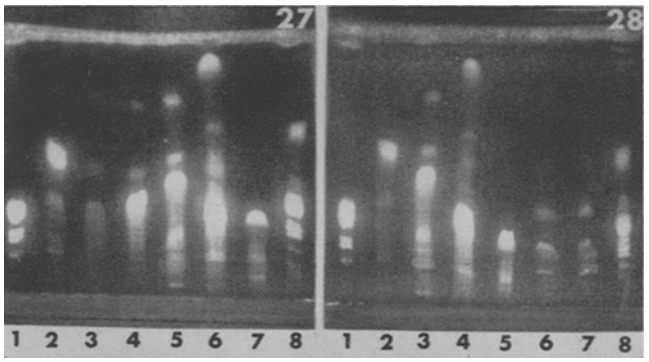


FIG. 27. Thin layer chromatogram illustrating the composition of fractions eluted from a DEAE column after application of beef liver lipid. The chromatogram was developed with chloroform/methanol/water (65/25/4, v/v/v) and sprayed with alkaline Rhodamine 6G reagent. 100 μg of each fraction was applied as a series of small spots. Spot 1 shows the composition of the chloroform/methanol 9/1 eluate with neutral lipid at the solvent front (not clearly distinguished from impurities migrating to the solvent front) followed by lecithin, sphingomyelin, and trace of material migrating in the lysolecithin region, but not lysolecithin as shown by paper chromatography. Spot 2 is from the first portion of the chloroform/methanol 7/3 eluate; behind the major component (phosphatidyl ethanolamine) are several minor spots of uncharacterized lipid. The tailing portion of the chloroform/methanol 7/3 eluate (spot 3) contained only a trace of phosphatidyl ethanolamine and more of the uncharacterized lipid. Spot 4 was prepared from the methanol eluate and showed a large spot of the uncharacterized component eluted in part with chloroform/methanol 7/3. Spot 5, a series of substances (uncharacterized) was eluted with chloroform/acetic acid 3/1. Spot 6 is from acidic lipids eluted with chloroform/acetic acid 3/1 containing 0.1 M ammonium acetate. The fastest moving component is cardiolipin. A number of uncharacterized substances are present and one of the major components is phosphatidyl inositol not clearly distinguished from other lipids. Spot 7, prepared from the acetic acid eluate, shows one major component clearly. This was demonstrated by paper chromatography to be a new, uncharacterized lipid. Spot 8, from total beef liver lipid, shows from above down: phosphatidyl ethanolamine, a new uncharacterized lipid migrating just ahead of lecithin, lecithin, sphingomyelin (2 spots overlapping with phosphatidyl inositol), and uncharacterized substances. Note the apparent simplicity of liver lipid before column chromatography. See text for additional details.

FIG. 28. A thin layer chromatogram (prepared as described in Figure 27 using chromatographic fractions from the same column) to indicate the variability of the thin layer chromatographic technique on the same day and to show 2 additional very minor fractions. 100 μg of each fraction was applied. Spot 1 is the chloroform/methanol 9/1 eluate; spot 2, the first portion of the chloroform/methanol 7/3 eluate; spot 3, the chloroform/acetic acid 3/1 eluate; spot 4, the chloroform/acetic acid 3/1 containing 0.1 M ammonium acetate eluate; spot 5, the glacial acetic acid eluate; spot 6, the chloroform/methanol 4/1 + 20 ml/liter concentrated aqueous ammonia eluate (a very minor fraction containing only trace of uncharacterized lipid); spot 7, another minor fraction containing only traces of uncharacterized lipid eluted with chloroform/methanol 4/1 containing 20 ml/liter concentrated aqueous ammonia and 0.05 M ammonium acetate; and spot 8, whole beef liver lipid. The composition of the fractions is as indicated for Figure 27. See text for additional information.

TABLE III

Elution of Anemone Lipid from DEAE

Fraction Nos.	Solvent	Lipid Class(es)	Weight (gm)	% Total Lipid
7– 15	C/M 9/1	Lecithin and nonionic lipid	0.17996	38.3
16– 19	C/M 9/1	5 uncharacterized components	0.01314	2.8
20– 42	C/M 7/3	Phosphatidyl ethanolamine and ceramide aminoethylphosphonate	0.06952	14.8
43– 50	C/M 7/3	Primarily ceramide aminoethylphosphate + some phosphatidyl ethanolamine	0.07167	15.2
51– 65	C/M 7/3	Uncharacterized ninhydrin positive lipid	0.00995	2.1
66– 81	CH_3OH	Uncharacterized lipid	0.02845	6.1
82–101	C/HAc 3/1	Pigments	0.00950	2.0
102–128	C/HAc 3/1 + 0.01 M NH_4Ac	Almost pure phosphatidic acid	0.01535	3.3
129–131	HAc	"Phosphatidyl serine" and uncharacterized lipid	0.04972	10.6
132–148	HAc	Pigments	0.00386	1.5
149–183	C/M 4/1 + 10 ml conc. aqueous NH_3 per liter	"Phosphatidyl inositol" and uncharacterized lipids	0.01310	2.8
			0.46422	99.5%

C = chloroform, M = methanol, HAc = acetic acid, NH_4Ac = ammonium acetate.

trate several important points. It is clear that the newer methods of analysis give strikingly different results from some of the older nonchromatographic methods, and that the newer methods developed originally for brain lipids can be modified for use with other samples as previously postulated (6). The value of DEAE cellulose column chromatography and paper chromatography as a means for examining a complex lipid mixture prior to undertaking other column separations is well illustrated by these studies. Using column chromatographic methods a new phospholipid was isolated and shown to contain a carbon to phosphorus bond. This appears to be the first reported instance of the natural occurrence of a sphingolipid with a free amino group and the first isolation of a pure phospholipid with a carbon-phosphorus bond.

Collection of sea anemone. The sea anemone, *Anthopleura elegantissima,* was collected off the shores of LaJolla, California by James Kittredge. The anemones were freed of as much loose extraneous material as possible, frozen over dry ice as a cake approximately ½ in. thick, and stored in the frozen state (−20C) for approximately 2 months prior to extraction.

Extraction of lipid. The frozen cakes were partly thawed, some sea shells and sand removed, and 60 g (wet weight) was extracted with 1220 ml of chloroform/methanol 2/1 by grinding for 5 min at medium speed in a Waring blender. The anemones were difficult to fragment by this procedure and the floating mass in the solvent was removed, cut into small pieces with a pair of scissors, and reground in the blender. The solid material was removed by filtration through a coarse grade sintered glass filter and the chloroform/methanol extract was evaporated to dryness at about OC in a rotary evaporator as described above. All of the work was carried out as much as possible under nitrogen. A highly precise quantitative determination of the total lipid content of the anemone was not feasible because of the impossibility of removing all sand and fragments of shells from the sample. The single chloroform/methanol extract used should contain at least 95% of the total lipid.

TABLE IV

Recovery of Anemone Lecithin from Silicic Acid

Fraction No.	Solvent	Wt (mg)	% Sample	Lipid class(es)
1	C/M 9/1	32.3	18.9	Nonionic (neutral) lipids and pigments
2	C/M 9/1	5.4	3.2	Nonionic (neutral) lipids and pigments
3	C/M 9/1	7.5	4.4	Nonionic (neutral) lipids and pigments
4	C/M 9/1	16.2	9.5	Nonionic (neutral) lipids and pigments
5	C/M 9/1	50.0	29.3	Nonionic (neutral) lipids and pigments
6	C/M 9/1	6.1	3.6	Nonionic (neutral) lipids and pigments
7	CH_3OH	52.4	30.7	Lecithin
	Total	169.9	99.6	

C = chloroform, M = methanol

The lipid extracted with chloroform/methanol was spread very evenly over the entire surface of a 2-liter round bottom flask after evaporation on the rotary evaporator. This very evenly spread material was then thoroughly dried in a vacuum desiccator over KOH and extracted with chloroform by swirling with 5–10 ml portions of chloroform very gently in the flask with care to be certain that chloroform wet all parts of the flask and its contents. The progress of lipid extraction was easily followed by the disappearance of color from the insoluble residue. The anemone lipid extract contained a number of highly colored substances and chloroform was used in small portions until the insoluble residue was white. The chloroform solution was filtered through a loose, prewashed (methanol and chloroform) glass wool plug to remove a small amount of insoluble matter dislodged from the side of the flask during extraction, and the extract was evaporated to dryness under nitrogen on a vacuum rack as described above. The lipid was then thoroughly dried over KOH: the final weight was 0.9399 g.

The residue, after extraction with chloroform, was then extracted with about 25 ml of 10% methanol in chloroform in order to determine the completeness of lipid removal. After filtration to remove insoluble material and evaporation on the vacuum rack, a residue weighing 0.1681 g was obtained. This was examined by paper chromatography and found to contain only traces of lipid. The chloroform extract can thus be considered to represent virtually the entire lipid extractible from the anemones. The total lipid content of the sample of sea anemone was determined as 1.56% of the fresh weight.

Column chromatographic fractionation. The major objectives of the present study were to examine the ninhydrin positive components of the sea anemone for the possible presence of 2-aminoethylphosphonic acid as an hydrolysis product, and to check for the presence of sphingomyelin. Method one involving DEAE column chromatography followed by paper chromatographic examination of the fractions was carried out first and then silicic acid column chromatography was chosen for the separation of the two fractions of major interest into some of the individual components.

A solution of 0.46655 g of anemone lipid in 6 ml of chloroform/methanol 9/1 was transferred to the "just dry" top of a DEAE cellulose column (2.5 × 20 cm) prepared as described above in chloroform/methanol 9/1, and the sample was thoroughly washed into the bed with the same solvent. Ten ml fractions were collected at a flow rate of approximately 3 ml/min maintained by turning the Teflon stopcock at the end of the column to the correct position. The

first fraction was collected with chloroform/methanol 9/1, and then the following solvents were used: chloroform/methanol 7/3, methanol, chloroform/glacial acetic acid 3/1, chloroform/glacial acetic acid 3/1 plus 0.01 M ammonium acetate, methanol wash to remove excess salt (approxamtely 5 column volumes or 350 ml of solvent), glacial acetic acid, another methanol wash (approximately 5 column volumes) to remove glacial acetic acid, and finally chloroform/methanol 4/1 containing 10 ml/liter of concentrated aqueous ammonia. The progress of the elution in each case was followed with the simple solids test using a 1 ml aliquot from each fraction (6) and solvent changes were made when solids could no longer be detected. Ninhydrin tests were carried out with 0.1 ml aliquots of the fractions (6). With each solvent change a new pigment fraction was eluted along with other lipid components. Most of the pigment components were present in minute amounts, but their intense color made them easy to detect during collection of the fractions.

The two fractions of major interest from the DEAE column were the chloroform/methanol 9/1 eluate that should contain all of the nonionic lipids as well as lecithin and sphingomyelin, and the fraction eluted with chloroform/methanol 7/3 that should contain phosphatidyl ethanolamine and any analogue containing 2-aminoethylphosphonic acid. These two fractions were subjected to silicic acid column chromatography after paper chromatographic examination disclosed the general nature of the components. Paper chromatography clearly indicated that lecithin was the only phospholipid present in the chloroform/methanol 9/1 eluate and therefore a simple silicic acid column procedure was feasible.

A 100-g portion of Mallinckrodt silicic acid for chromatography (100 mesh) was washed with methanol, chloroform/methanol 1/1, and finally chloroform on a coarse grade sintered glass filter under gentle suction from a water pump, and enough of the silicic acid slurry in chloroform was passed into a 2.5 × 40 cm chromatography tube equipped with a Telflon stopcock to give a column height of 20 cm. A solution of 0.17086 g of the chloroform/methanol 9/1 eluate from the DEAE column was applied to the silicic acid column in chloroform and the sample was washed in thoroughly with chloroform. Approximately 35 ml of chloroform (1 column volume about 70 ml) was passed through the column. It became apparent that some of the pigment would be eluted very slowly with chloroform and 10% methanol in chloroform was applied to the column. This solvent brought the pigments off very rapidly and a rather crude fractionation on the basis of different colored pigments was made to give six pigment fractions (all eluted with chloroform/methanol 9/1). Lecithin plus a trace of other pigments was then eluted from the column with methanol. The elution was carried out until the solids test was negative with a 1-ml aliquot. Fractions (10 ml) were collected at a flow rate of approximately 3 ml/min maintained by application of slight nitrogen pressure.

The ninhydrin positive material eluted with chloroform/methanol 7/3 from DEAE was applied to the same silicic acid column used for the fractionation just described. The silicic acid bed was first washed with three volumes of methanol and methanol was replaced by chloroform/methanol 9/1. Washing was carried out under enough nitrogen pressure to give a flow rate of approximately 10 ml/min. The sample

TABLE V

Sea Anemone Ninhydrin Positive Components Separated on Silicic Acid

Fraction (No.)	Solvent	Wt (mg)	% Sample	Lipid class(es)
1– 5	C/M 9/1	0	0	None
6– 32	C/M 9/1 and 85/15	32.2	28.8	Uncharacterized
33– 62	C/M 85/15	35.9	32.1	Phosphatidyl ethanolamine
63– 72	C/M 85/15	3.1	2.7	Phosphatidyl ethanolamine
73– 78	C/M 85/15	0	0	No lipid
79–100	C/M 85/15	34.3	30.6	Ceramide aminoethylphosphonate
Bulk (200 ml)	CH3OH	5.9	5.2	Uncharacterized
	Total	111.4	99.4	

C = chloroform, M = methanol

weighing 0.1120 g dissolved in 10 ml of chloroform/methanol 9/1 was applied to the top of the silicic acid column and the sample washed in with the same solvent. Fractions (10 ml) were collected in glass stoppered tubes at the rate of 3 ml/min. The first 22 fractions were collected using 10% methanol in chloroform as eluting solvent, and then 15% methanol in chloroform was applied to the top of the column and 88 more fractions collected. Finally methanol was added and a 200 ml bulk fraction was collected.

Two completely separated major ninhydrin positive fractions were eluted from the silicic acid column. There were six tubes between the two major fractions that did not contain lipid so that the separation was complete. Two other fractions, one eluted rapidly ahead of the ninhydrin positive material and one cleared from the column with methanol, were not examined in detail.

Table III shows the amount of material eluted with each solvent from the DEAE column run. Total recovery was satisfactory. Table IV shows the results of the fractionation on silicic acid of the chloroform/methanol 9/1 eluate from the DEAE column, again with satisfactory recovery of the sample applied. Table V shows the results obtained by silicic acid column fractionation of the ninhydrin positive fraction from the DEAE column (recovery also quite satisfactory). Table VI summarizes the data obtained from the three runs giving the percentages of the various fractions and the probable constituents.

Three substances were isolated in pure form as shown by paper chromatography and infrared spec-

TABLE VI

Composition of Sea Anemone Lipid

	Lipids	Percent
1)	Total nonionic lipid (including pigments, sterols, and glycerides)	24.2
2)	Lecithin	14.4
3)	5 uncharacterized components (eluted from DEAE between lecithin and phosphatidyl ethanolamine)	2.8
4)	Uncharacterized (eluted from DEAE with phosphatidyl ethanolamine, but eluted from silicic acid before phosphatidyl ethanolamine)	8.8
5)	Phosphatidyl ethanolamine	10.6
6)	Ceramide aminoethylphosphonate	9.3
7)	Uncharacterized (eluted from DEAE with phosphatidyl ethanolamine and from silicic acid with methanol)	1.6
8)	One uncharacterized ninhydrin positive component eluted after (6) from DEAE with C/M 7/3	2.1
9)	Uncharacterized (eluted from DEAE with methanol)	6.1
10)	Pigment eluted from DEAE with C/HAc 3/1	2.0
11)	Eluted from DEAE with C/HAc/salt, presumably phosphatidic acid	3.3
12)	Lipid eluted from DEAE with HAc ("phosphatidyl serine" and uncharacterized lipids)	10.6
13)	"Phosphatidyl inositol" and uncharacterized lipids (eluted from DEAE with C/M/NH3)	2.8
		98.6%

C = chloroform, M = methanol, HAc = acetic acid

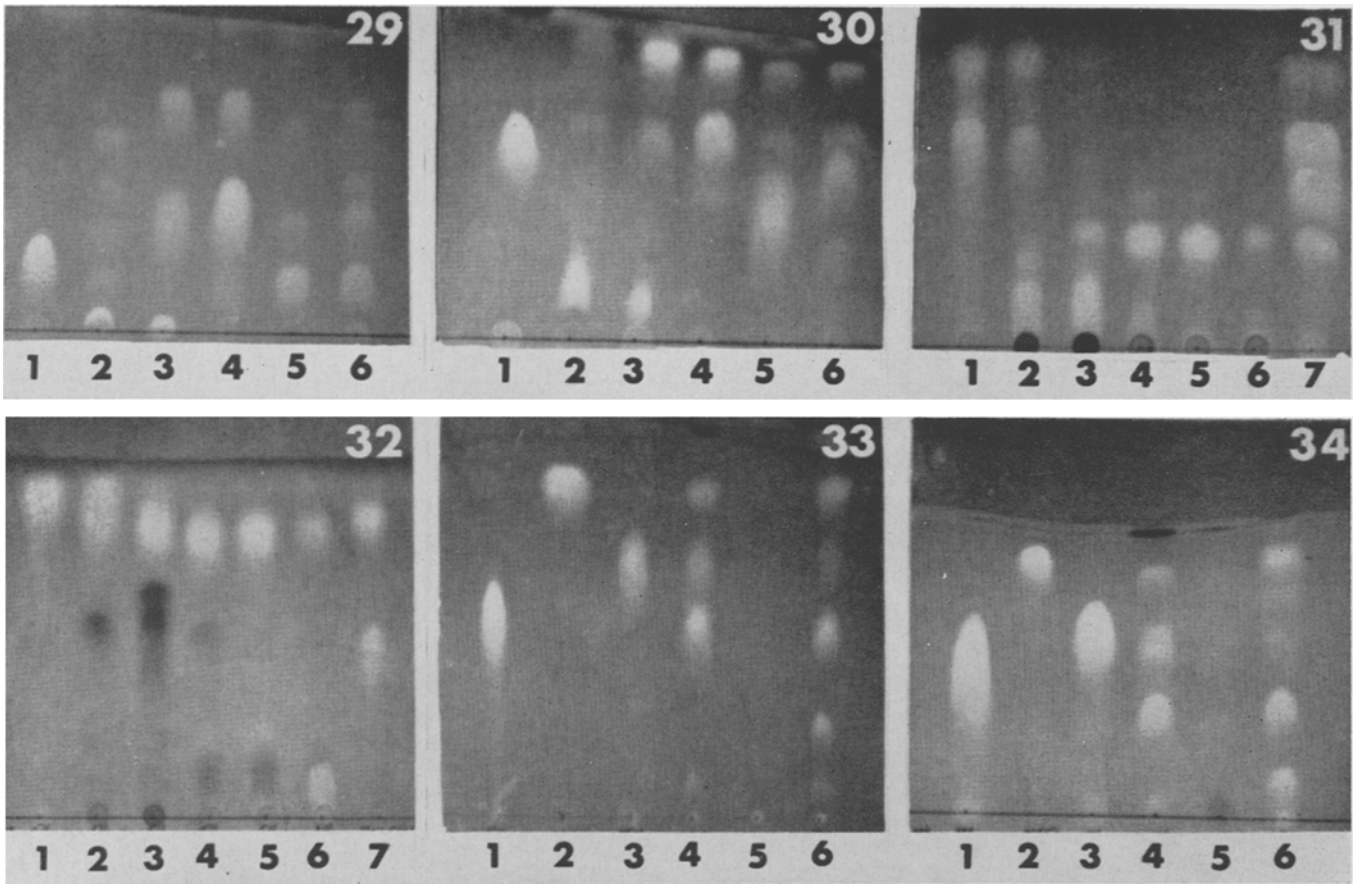


FIG. 29. Paper chromatogram showing the composition of some fractions eluted from a DEAE cellulose column after appplication of sea anemone lipid. The chromatogram was developed with chloroform/acetone/acetic acid/water mixture (system 15, ref. 5) and dipped in Rhodamine 6G reagent (100 μg each fraction spotted). Spot 1 is from a chloroform/methanol 9/1 eluate that contained nonionic (neutral) lipids at the solvent front and lecithin as the only phospholipid component (sphingomyelin entirely absent). Spot 2, a very minor fraction eluted with additional chloroform/methanol 9/1, contained largely uncharacterized lipids. Spot 3, the first portion of the chloroform/methanol 7/3 eluate, contained from above downward: phosphatidyl ethanolamine, ceramide aminoethylphosphonate and uncharacterized material at the origin. Spot 4, from the second portion of the chloroform/methanol 7/3 eluate, shows enrichment with respect to ceramide aminoethylphosphonate and the presence of an additional component migrating just behind ceramide aminoethylphosphonate. Spot 5, from the final portion of the chloroform/methanol 7/3 eluate, shows only very small amounts of phosphatidyl ethanolamine and ceramide aminoethylphosphonate and more of the minor uncharacterized ninhydrin positive lipid eluted partially in the previous fraction. Spot 6, from total anemone lipid, shows the major components from above down: neutral (nonionic) lipid at the solvent front followed by phosphatidyl ethanolamine, phosphatidyl serine, ceramide aminethylphosphonate, and lecithin. Many of the minor components are not seen in the crude lipid mixture. Some migrate with the major components while others are below the limit of detectibility.

FIG. 30. The same fractions from the DEAE column described in the legend for Figure 29, but the chromatogram was developed with chloroform/methanol/aqueous ammonia (system 12, ref. 5). Note the uncharacterized component in spots 2 and 3 that migrates just off the origin.

FIG. 31. Paper chromatogram developed with 5% ether in hexane on acidic silicic acid paper (system 3, ref. 5) to show some of the neutral lipid components of the sea anemone. The fractions were eluted with chloroform/methanol 9/1 from a silicic acid column (see text). The sample was the chloroform/methanol 9/1 eluate from a DEAE column. No attempt was made to get clean separations of neutral lipids (an arbitrary series of cuts based on different pigment colors was made) since the column was used for the isolation of pure lecithin (see Figs. 33 and 34). 50 μg of each of six consecutive neutral lipid fractions was applied. Spot 1 shows components in the correct position for sterol esters and glycerides with some uncharacterized material, spot 2 contained the same substances as spot 1 plus a small amount of free sterol and much more polar components migrating in the mono and diglyceride regions, while spots 3, 4, 5, and 6 showed primarily sterol and substances not migrating from the origin. Spot 7 was prepared from 20 μg each of (from above downward) cholesterol palmitate, beef spleen triglyceride, oleic acid, and cholesterol. See text for additional comments.

FIG. 32. Paper chromatogram developed with 60% ether in hexane from the same fractions described in the legend for Figure 31. This chromatogram shows the migration of pigmented substances that give dark purple spots on chromatograms. The controls at the far right (10 μg each) are di and monoglycerides. The pigments of the sea anemone can be separated into a number of spots with system 7 (5).

FIG. 33. A glass fiber impregnated paper chromatogram developed with chloroform/acetone/acetic acid/water mixture (solvent 15, ref. 5) to be compared with Figure 34 using the same solvent system but impregnated cellulose paper. Spots 1, 2, and 3 show the pure lipid fractions isolated by column chromatography from sea anemone lipid (see text) and their migration with respect to total anemone and brain lipids. Spot 1 was from 50 μg of lecithin, spot 2 from 100 μg phosphatidyl ethanolamine, and spot 3 from 50 μg of ceramide aminoethylphosphonate. Spot 4, from 100 μg of crude anemone lipid, clearly shows the three major components that were isolated in pure form. Spot 5 prepared from 100 μg of the chloroform/methanol 9/1 extract of a residue left after chloroform extraction of the crude anemone lipid (see text) to illustrate the absence of lipids in the residue. Spot 6, from 80 μg of beef brain lipid, shows from above downward cholesterol, cerebroside plus phosphatidyl ethanolamine, a very faint spot of cerebroside containing hydroxy fatty acid, phosphatidyl serine, lecithin, sphingomyelin, and phosphatidyl inositol plus sulfatide. The purity of the main lipid components of the anemone is clearly shown by this paper chromatogram.

FIG. 34. Paper chromatogram developed with the same solvent mixture described for Figure 33 but using cellulose impregnated paper rather than glass fiber impregnated paper. Fractions are as indicated in the legend for Fig. 33. The purity of the three main lipid fractions isolated by column chromatography (spots 1, 2, and 3) is again indicated by this chromatogram. Note the very minor component migrating just behind ceramide aminoethylphosphonate (spot 3). See text for further comments.

troscopy. These three substances represented 34.3% of the total lipid. The other fractions were mixtures and were not further separated in the present study, although paper chromatographic examinations were carried out to indicate the degree of complexity of the fractions. The only other fraction that appeared to be essentially one substance was the chloroform/acetic acid/salt eluate from the DEAE column (3.3% of the total lipid) that is apparently phosphatidic acid. The fact that the lipid was eluted with the proper solvent from DEAE columns and migrated as phosphatidic acid in different paper chromatographic systems indicates with a high degree of certainty that the substance is in fact phosphatidic acid (trace amounts of other lipid materials were detectible on the paper chromatograms).

Paper chromatography of anemone lipid. Some of the paper chromatographic data with the column fractions are shown in Figures 29–36 and the legends for the figures explain the composition of the fractions from the columns. The purity of the lecithin, phosphatidyl ethanolamine, and ceramide aminoethylphosphonate is shown in Figures 33 and 34. Figures 35 and 36 prepared from chromatograms sprayed with ninhydrin reagent show clearly that there are four ninhydrin positive components in the original anemone lipid extract. One of these was recovered in pure form and shown to be phosphatidyl ethanolamine. The second, also isolated in pure form, is the new ninhydrin positive sphingolipid; the third substance is a minor component that is still uncharacterized, and the fourth appears to be phosphatidyl serine.

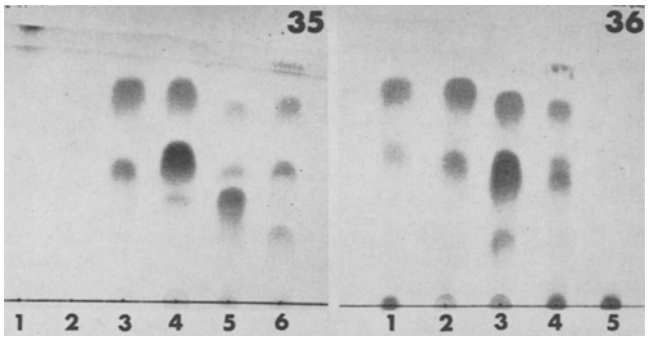


FIG. 35. Paper chromatogram prepared as described in the legend for Figure 29 but sprayed with ninhydrin to show the number of ninhydrin positive components in crude sea anemone lipid and in fractions from DEAE columns. Spots 1 and 2 eluted with chloroform/methanol 9/1 did not contain ninhydrin positive substances, but spot 3 showed clearly phosphatidyl ethanolamine above and ceramide aminoethylphosphonate below. Spot 4 was enriched with respect to ceramide aminoethylphosphonate and showed the presence of a small amount of another ninhydrin positive component in this intermediate fraction, spot 5 was greatly enriched with respect to the minor component migrating just behind ceramide aminoethylphosphonate and eluted somewhat less readily from DEAE with chloroform/methanol 7/3. The 6th spot, from crude sea anemone lipid, showed from above downward phosphatidyl ethanolamine, ceramide aminoethylphosphonate, an almost undetectible trace of a very minor component and phosphatidyl serine.

FIG. 36. Paper chromatogram prepared with system 15 (see ref. 5) using chloroform/acetone/acetic acid/water as solvent and sprayed with ninhydrin reagent after development. Spot 1 shows phosphatidyl ethanolamine and phosphatidyl serine in total beef brain lipid and free amino acids at the origin. Spot 2 shows the first portion of the chloroform/methanol 7/3 eluate from DEAE contaoining phosphatidyl ethanolamine and ceramide aminoethylphosphonate, and spot 3 shows the presence of a somewhat *more* polar minor component eluted in the middle portion of the chloroform/methanol 7/3 *eluate*. Spot 4, from the chloroform extract of anemone lipids, shows from above downward: phosphatidyl ethanolamine, phosphatidyl serine, ceramide aminoethylphosphonate, a small amount of uncharacterized ninhydrin positive lipid, and free amino acids (at the origin). Spot 5 shows the chloroform/methanol 9/1 extract of the residue left after chloroform reextraction. It is apparent that essentially no ninhydrin positive lipid is present in this extract (free amino acids present at the origin). Compare the results with the chromatograms stained with Rhodamine 6G shown in Figures 29 and 34.

Infrared spectra of anemone lipids. Figures 37,38, and 39 show the infrared spectra from the pure lecithin, phosphatidyl ethanolamine, and the new lipid. Lecithin isolated from the sea anemone gives a spectrum almost identical to that obtained from brain lecithin (compare Figs. 8 and 37). Similarly, phosphatidyl ethanolamine from the anemone gives a spectrum similar to the phosphatidyl ethanolamine isolated from beef brain (compare Figs. 9 and 38), but distinctly different from the spectrum of synthetic dimyristoylphosphatidyl ethanolamine (Fig. 11). Lecithin and phosphatidyl ethanolamine from the anemone were strongly Feulgen positive indicating a very large amount of the plasmalogen form of each of these lipids. This indication was borne out by the demonstration of fatty aldehydes by paper chromatography of the hexane soluble portion of acid hydrolysates of both preparations.

The spectrum prepared from the new ninhydrin positive lipid is clearly not that of a glycerol phosphatide since the characteristic ester band at 5.70–5.75 μ is not present. Instead, absorption characteristic of amide linked fatty acid is present.

The column and paper chromatographic behavior as well as the infrared spectra indicated that lecithin and phosphatidyl ethanolamine isolated from the sea

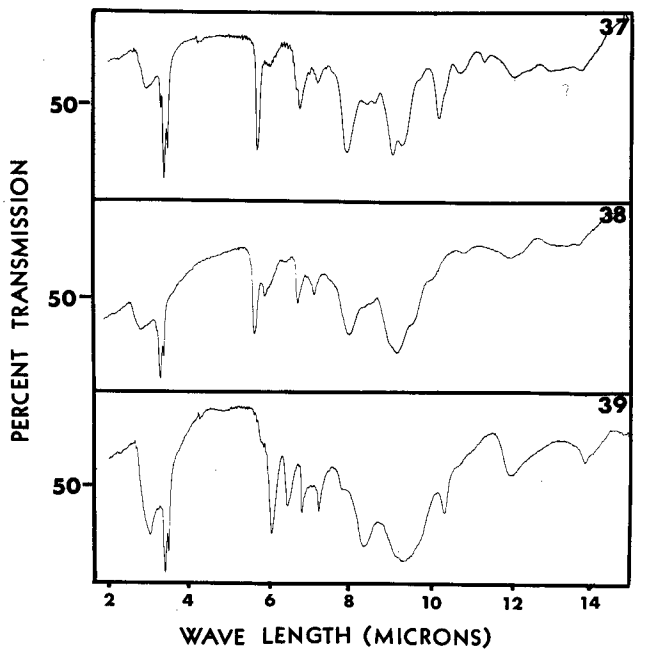


FIG. 37. Infrared spectrum of pure lecithin isolated from sea anemone lipid by column chromatography (see text). The spectrum was obtained as a film on a silver chloride plate. Compare with the spectrum of beef brain lecithin (Fig. 8).

FIG. 38. Infrared spectrum of phosphatidyl ethanolamine isolated from the sea anemone (see text). The spectrum was obtained from a KBr pellet *containing* 1.49% lipid. Compare with the spectrum of brain phosphatidyl ethanolamine (Fig. 9).

FIG. 39. Infrared spectrum of ceramide aminoethylphosphonate isolated as described in the text and examined as a KBr pellet containing 1.50% lipid. Note the absence of the typical ester band at 5.70–5.75 μ and the presence of typical amide bands at 6.06 and 6.45 μ. Absorption at 10.37 μ is related in part to the *trans* double bond of sphingosine and allied substances (Figs. 42 and 43).

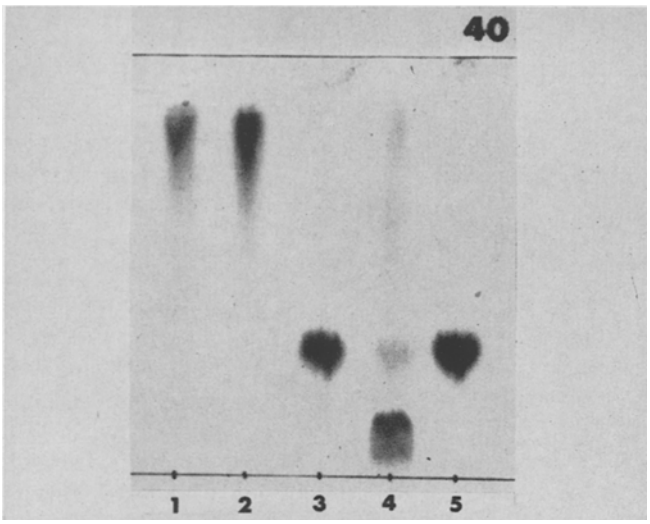


FIG. 40. Chromatogram run on Whatman 3 MM paper developed with butanol/acetic acid/water (4/1/5, v/v/v, upper layer) and sprayed with ninhydrin. Spot 1 is from 60 μg of phosphatidyl ethanolamine and spot 2 from 60 μg of ceramide aminoethylphosphonate from the sea anemone prior to hydrolysis. The lipids are at the solvent front and ethanolamine and 2-aminoethylphosphonic acid are absent before hydrolysis. Spot 3 shows the ethanolamine present in 15 μg of water soluble hydrolysis products from phosphatidyl ethanolamine of the anemone. Spot 4, from 20 μg of water soluble hydrolysis products of ceramide aminoethylphosphonate, shows 2-aminoethylphosphonic acid as the major spot. It also shows minor components migrating with higher R_f values (see text for details) and a trace of ethanolamine. Spot 5 is from 20 μg of water soluble hydrolysis products from synthetic phosphatidyl ethanolamine with a single spot from ethanolamine.

anemone were similar to the lecithin and phosphatidyl ethanolamine of beef brain with the exception that the lecithin fraction from beef brain is virtually devoid of plasmalogen while the lecithin from the anemone is rich in the plasmalogen form.

A good deal of information was available at this stage with regard to the composition of the new ninhydrin positive lipid. It was evident that it had the same proportion of ionic groups as phosphatidyl ethanolamine (from DEAE and silicic acid chromatography). One of these groups was evidently the $-NH_3$ group (ninhydrin positive). The ion exchange behavior of zwitterion lipids of this type has been discussed previously (6). No definite indication of a carbon-phosphorus bond was obtained by these preliminary examinations. It was evident, however, that the new lipid was not a glycerol containing compound, but rather that fatty acid was amide linked. This finding aided considerably in setting up the hydrolysis conditions for the new lipid.

Hydrolysis of anemone lipids. The three pure lipid fractions from the anemone and a control sample of synthetic dipalmitoylphosphatidyl ethanolamine were then hydrolyzed with acid. In each case a known amount of lipid was transferred to a Pyrex glass tube in chloroform/methanol 2/1, the solvent evaporated under a current of pure nitrogen, 3 ml of 2 N HCl added, nitrogen blown into the tube to replace any air, and the tube sealed. Hydrolysis was then carried out at 100C for the desired time. The tubes were cooled, opened, and redistilled n-hexane added in 3-ml portions and mixed vigorously with the tube contents until no more lipid floating over the aqueous HCl or sticking to the sides of the tubes was apparent. This required three extractions in each case with 3 ml of n-hexane. (Pure sphingosine hydrochloride or phytosphingosine hydrochloride are not very soluble in hexane, but the lipid portion from the aqueous acid hydrolysates of sphingolipids dissolves in this solvent. Apparently the presence of fatty acid along with the sphingosine and similar substances facilitates the extraction.) In the case of the new ninhydrin positive lipid from the anemone it was necessary to shake the hexane with the lipid floating on the aqueous acid phase for several minutes to effect solution. The hexane extracts were then evaporated to dryness under nitrogen and weighed. The aqueous acid portion of each hydrolysate was evaporated to dryness under a stream of nitrogen on a vacuum rack, treated with 3–5 ml of water, frozen, and lyophilized to remove the last traces of HCl. The samples were dried over KOH, weighed, and water and chloroform/methanol 2/1 used to prepare solutions of known concentrations for the aqueous and lipid phases respectively.

Phosphatidyl ethanolamine from the anemone was hydrolyzed using 12.05 mg of sample and a 4-hr hydrolysis period. After hydrolysis 6.08 mg of hexane soluble and 6.28 mg of water soluble material were recovered. The hydrolysis of synthetic dipalmitoylphosphatidyl ethanolamine was carried out using 14.60 mg of lipid heated for 6 hr to give 10.31 mg of hexane soluble and 7.85 mg of water soluble material. The new lipid was hydrolyzed using 11.98 mg of material heated for 21 hr to give 8.14 mg of hexane soluble and 5.91 mg of water soluble material.

Synthetic phosphatidyl ethanolamine gave 53.6% and anemone phosphatidyl ethanolamine 53.0% of the starting weight of the sample as water soluble material. (Total recovery after acid hydrolysis is invariably above 100%. It is higher than expected from simple hydrolysis equations since the amount of water retained by hydrolysis products is greater than that retained by the lipid.) The close correspondence of the percentage recoveries in the water soluble and hexane soluble portions of the hydrolysates from synthetic phosphatidyl ethanolamine and that isolated from the anemone is strong indication of the presence of only phosphatidyl ethanolamine of the type normally found in mammalian tissues. This was further confirmed by paper chromatography on Whatman 3 MM paper using butanol/acetic acid/water (4/1/5, v/v/v, upper phase) as solvent. The chromatogram was sprayed with ninhydrin and as shown in Figure 40 the anemone phosphatidyl ethanolamine prior to hydrolysis did not contain the substances released on hydrolysis. After hydrolysis, both the anemone and synthetic phosphatidyl ethanolamines gave a typical spot for ethanolamine. It is thus apparent that no 2-aminoethylphosphonic acid was obtained from the phosphatidyl ethanolamine fraction of the anemone since this substance migrates well behind ethanolamine. Kittredge et al. (29) established that 2-aminoethylphosphonic acid is stable to strong HCl and it would not be hydrolyzed under the conditions used. Hydrolysis of a phosphonic acid analogue of phosphatidyl ethanolamine should have released 2-aminoethylphosphonic acid.

Paper chromatography of the new sphingolipid aqueous hydrolysis products showed (Fig. 40) that, while the original substance before hydrolysis did not contain material migrating on paper chromatograms in the ethanolamine or 2-aminoethylphosphonic acid regions, after hydrolysis the major spot in the water soluble portion of the hydrolysate migrated

like 2-aminoethylphosphonic acid. Light spots for ethanolamine and some lipid components (with higher R_f values) not removed completely from the water soluble hydrolysis products can be seen. The solution of water soluble hydrolysis products frothed when shaken, although it was entirely clear. Evidence for the presence of a small amount of lipid in the aqueous fraction was obtained by paper chromatography with lipid solvents.

The hexane soluble portion from the new sphingolipid was strongly ninhydrin positive and when examined by paper chromatography using the alkaline paper systems with chloroform/methanol 98/2 and 95/5 (systems 20 and 21, Table I) showed the presence of components migrating in the general regions of sphingosine and phytosphingosine (Figs. 41,42). The migration of phytosphingosine anhydro base, phytopshingosine, the hydrolysis products from the anemone sphingolipid, sphingosine isolated from beef brain, a mixture of sphingosine and dihydrosphingosine, and a preparation of O-methyl sphingosine containing a small amount of sphingosine are all shown in Figures 41 and 42. It is to be noted that most of the preparations showed more than one spot. The major spots in each case are the compounds listed.

It is apparent from the chromatograms that an additional hydroxyl group on phytosphingosine as compared to sphingosine produces a marked decrease in paper chromatographic migration, and further that the loss of an hydroxyl group from sphingosine as in the formation of O-methyl sphingosine produces a similar large increase in migration. The light spots between these various major components indicate that substances of different chain length and/or degree of unsaturation are present in the samples. It is expected that an increase in chain length and/or an increase in unsaturation should increase migration while a decrease in chain length or unsaturation should decrease migration. In the case of hydrolysates from sphingolipids it is expected that the natural erythro forms would have been converted in part to the corresponding threo forms. Examination of erythro and theo sphingosines has shown these to be readily separable in both solvent systems (Figs. 41,42). The paper chromatographic examinations demonstrate the presence of erythro and threo sphingosines as well as some erythro and threo phytosphingosines along with related compounds of different chain length and/or unsaturation. These substances are all ninhydrin positive.

Infrared examination of hydrolysis products of anemone lipids. A complete identification of 2-aminoethylphosphonic acid as an hydrolysis product of the new sphingolipid of the anemone was not possible with paper chromatography alone since 2-aminoethylphosphonic acid is not separated from ethanolamine-O-phosphate with the systems employed (29). Although the two substances can be distinguished by differences in chemical properties (29), we desired a more rapid and still unequivocal method applicable to very small amounts of material. Infrared examination was found suitable for this purpose. The general nature of this method is described above.

In the case of phosphatidyl ethanolamine from the sea anemone, the problem was to distinguish by infrared examination between the production of 2-aminoethylphosphonic acid and an equimolar mixture of glycerophosphate and ethanolamine. Although glycerol should be released from a phosphonic acid analogue of phosphatidyl ethanolamine under the hydrolytic conditions employed, glycerol would be lost during preparation of the sample and only 2-aminoethylphosphonic acid should be present. When the water soluble acid hydrolysis products from the phosphatidyl ethanolamine of the sea anemone were compared to those obtained from brain phosphatidyl ethanolamine and synthetic dipalmitoylphosphatidyl ethanolamine (or an equimolar mixture of glycerophosphate and ethanolamine treated in the same way) essentially identical spectra were obtained. A typical spectrum is shown in Figure 46 and can be compared to Figure 43 prepared from glycerophosphate and ethanolamine. Unless the standards are exposed to exactly the same conditions as the samples to insure the presence of similar ionic forms, water of hydration, and amounts of ethanolamine the spectra will not be identical, although the nature of the hydrolysis products can still be determined in some cases. The spectral data were in agreement with chromatographic data and gave an independent unequivocal answer: the phosphatidyl ethanolamine of the anemone did not contain a phosphonic acid group.

In the case of the new sphingolipid, either ethanolamine-O-phosphate or 2-aminoethylphosphonate, sphingosine (or similar bases) and fatty acids were expected after hydrolysis. The infrared spectrum of the hexane soluble products after hydrolysis matched well with spectra obtained from sphingosine hydrochloride and fatty acids treated in the same way. The infrared spectrum of the water soluble hydrolysis product was clearly that of 2-aminoethylphosphonic acid and

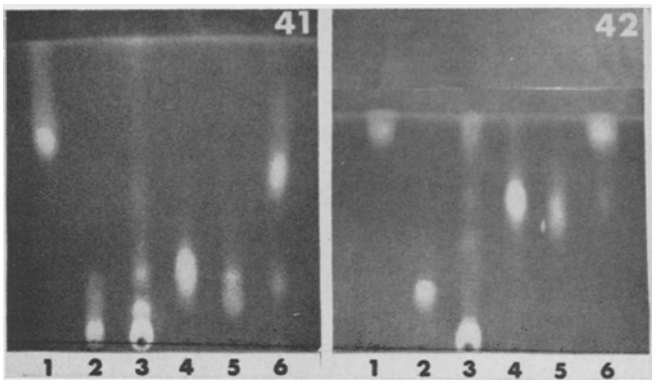


FIG. 41. Paper chromatogram developed with system 20 of Table I and stained with Rhodamine 6G reagent. Spot 1 was prepared from 25 μg of phytosphingosine anhydro base (gift from Paul O'Connell) and shows a single very bright spot with a number of other components migrating closer to the solvent front. Spot 2, prepared from 25 μg of phytosphingosine (gift from Paul O'Connell), shows again minor components migrating ahead of the main component, phytosphingosine, which migrates just off the origin and appears as a very bright spot. Spot 3 is from 100 μg of the hexane soluble portion of an acid hydrolysate of ceramide *aminoethylphosphonate* isolated from the sea anemone. A number of minor components are shown migrating throughout the body of the chromatogram; also shown are spots in the correct positions for sphingosine, dihydrosphingosine, and fatty acids (the bright spot at the origin). Spot 4, from 25 μg of a sphingosine preparation, shows principally a single large spot due to sphingosine. Spot 5 is from 25 μg of a preparation enriched with dihydrosphingosine but also contains sphingosine. Dihydrosphingosine migrates just behind sphingosine and overlaps slightly with it. Spot 6 is from 25 μg of a preparation enriched in O-methylsphingosine but contains a small amount of sphingosine (the minor slower-migrating component) and substances related to O-methylsphingosine migrating ahead of O-methylsphingosine.

FIG. 42. Paper chromatogram prepared using system 21 of Table I and spotted with the same preparations in the same order and amount as described in the legend for Figure 41. In this solvent system all of the lipids have somewhat greater R_f values.

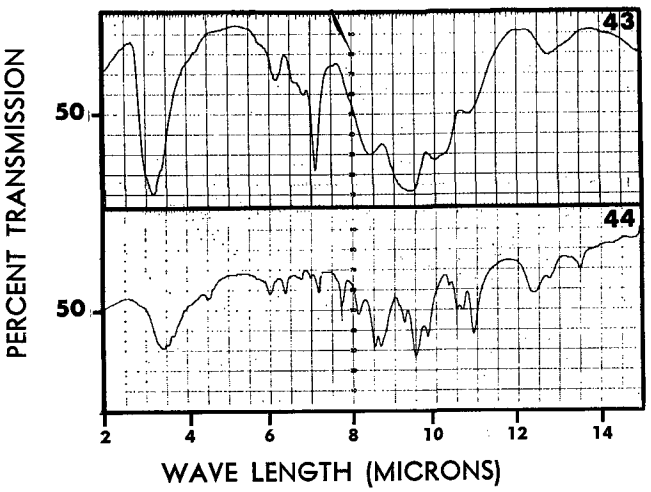
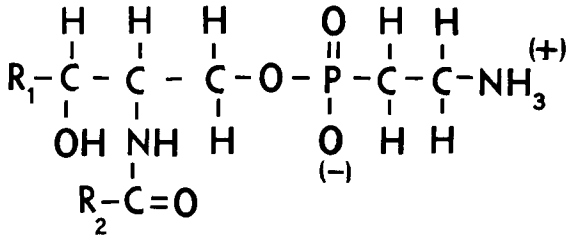


FIG. 43. Infrared spectrum obtained from an equimolar mixture of ethanolamine and sodium β-glycerophosphate dissolved in 2 N HCl and lyophilized before mixing with KBr to make a pellet 0.137 mm thick containing 2.21% sample. This mixture serves as a reasonably adequate control for the water soluble portion of a phosphatidyl ethanolamine acid hydrolysate. Compare with Figure 46.

FIG. 44. Infrared spectrum of 2- aminoethylphosphonic acid isolated from the sea anemone by James Kittredge and reproduced from Kittredge et al. (29) for comparison with Figure 47 obtained from an acid hydrolysate of ceramide aminoethylphosphonate isolated from the sea anemone as described in the text.

not ethanolamine-O-phosphate (compare Figs. 44,45, and 47).

Structure and significance of the new sphingolipid of the anemone. From the data presented above, the structure of the new lipid can be formulated as:



CERAMIDE AMINOETHYLPHOSPHONATE

where R_1 represents the carbon chain of sphingosine, phytosphingosine, etc. and R_2 represents the hydrocarbon chains of fatty acids. The rather descriptive name, ceramide aminoethylphosphonate, seems appropriate. The placement of the 2-aminoethylphosphonate group on the primary hydroxyl group is by analogy to other sphingolipids, although no direct data have been obtained on this point. The presence of a free amino group is certain and 2-aminoethylphosphonate has been positively identified as an hydrolysis product. A mixture of fatty acid and long chain bases has also been demonstrated.

The biological significance of ceramide aminoethylphosphonate is not known but it is evident that this ninhydrin positive sphingolipid completely replaces sphingomyelin which is so widely encountered in animal tissues. This conclusion is in direct contrast to the findings of Bergmann and Landowne who reported sphingomyelin as the major lipid of this same species. Our data are, however, clear and unambiguous: no sphingomyelin was present and other lipids were found. No trace of ceramide or cerebroside was seen in the anemone lipid mixture.

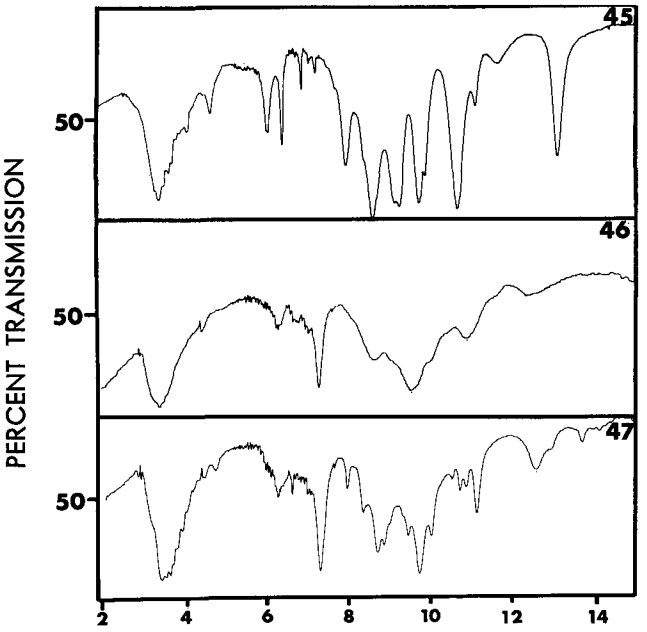


FIG. 45. Infrared spectrum of ethanolamine-O-phosphate prepared as a 0.137 mm thick KBr pellet containing 1.44% sample. This spectrum is clearly differentiated from that of 2-aminoethylphosphonic acid (Fig. 44) and from the water soluble acid hydrolysis product of ceramide aminoethylphosphonate (Fig. 47). See text for further comments.

FIG. 46. Infrared spectrum obtained from the water soluble portion of a 3 N HCl hydrolysate of synthetic dipalmitoylphosphatidyl ethanolamine as described in the text. The spectrum was prepared from a KBr pellet containing 1.55% sample. Note the similarity to an equimolar mixture of ethanolamine and glycerophosphate (Fig. 43). An almost identical spectrum was obtained from the water soluble hydrolysis products of sea anemone phosphatidyl ethanolamine.

FIG. 47. Infrared spectrum obtained from water soluble HCl hydrolysis products of ceramide aminoethylphosphonate as described in the text. The spectrum was prepared from a KBr pellet containing 1.50% sample. The marked similarity of this spectrum to that of 2-aminoethylphosphonic acid (Fig. 44) is evident. The major difference between the two spectra is the strong band at 7.18 μ in the hydrolysate of ceramide aminoethylphosphonate related to —NH absorption from HCl treatment. This band is very minor in the 2-aminoethylphosphonic spectrum shown in Figure 44 since the preparation was not treated with HCl prior to preparation of the spectrum. Other minor differences in the two spectra are also related to the fact that 2 slightly different ionic forms were used for the preparation of the spectra. The fact that 2-aminoethylphosphonic acid can be clearly identified even when the reference substance has not been treated in exactly the same manner as the sample is quite clear. The spectrum is entirely different from that of ethanolamine-O-phosphate (Fig. 45). See text for further comments.

The finding of Kittredge et al. (29) that 2-aminoethylphosphonic acid is present in hydrolysates of anemone lipid is confirmed and the lipid has been isolated and characterized.

The studies of Rajogopal and Sohonie (30) on the anemone *Gyrostoma sp.* were carried out with nonchromatographic methods. It is not surprising then that their results differ from ours. We did not detect cerebroside or sphingomyelin reported by these investigators as components of their anemone lipid extracts. Rapport and Alonzo (31) reported the presence of the typical α,β-unsaturated ether type of plasmalogen in a sea anemone and noted that this occurred in several invertebrates primarily as ethanolamine plasmalogen. In a short note from the same laboratory (32) a great deal of ethanolamine and

choline plasmalogen and a small amount of sphingomyelin was reported to be present in *Anthopleura elegantissima* on the basis of TLC examinations. The observation that *Anthopleura elegantissima* is rich in choline and ethanolamine plasmalogen is in keeping with our results. We did not detect sphingomyelin and a new lipid not reported by the other investigators was isolated and characterized. It is evident that TLC alone as used by Rapport and Alonzo did not distinguish sphingomyelin and ceramide aminoethylphosphonate from other lipids.

The great superiority of column chromatographic methods over paper chromatographic methods alone for the study of new lipid mixtures is well illustrated by a comparison of the results obtained in the present studies with those of Hack et al. (33) who studied the anemone but did not report new lipids and failed to gain any information of the type reported above.

The role of the phosphonate grouping and its metabolic fate is yet to be determined; however, we will conform to well established custom on the occasion of the discovery of a new ionic lipid and suggest without direct data that it may play a role in permeability.

Lipid Composition of Beef Brain

Table VII is a compilation of the lipid classes thus far isolated and characterized from beef brain and the best available estimates of the amounts of these lipid classes. The basis for the entries in the table and the problems in the analysis of beef brain lipid are discussed below. The values in Table VII differ in some cases from those reported previously (6). The present values were obtained with improved methods and are based on a larger number of determinations.

General objectives and definitions. We have attempted to isolate each lipid class quantitatively. A lipid class is defined here as a group of lipids having the same polar functional groups regardless of differences in fatty acid and/or fatty aldehyde composition. The cerebrosides containing normal and hydroxy fatty acids are grouped as one lipid class and the phosphatidyl ethanolamines containing two fatty acids or one fatty acid and a fatty aldehyde (ethanolamine plasmalogen) are termed together phosphatidyl ethanolamine. Sulfatides and ganglioside are more heterogeneous groups with subclasses in which differences are not confined to fatty acids. Sulfatides are considered as a single class that contains sulfate, and gangliosides are grouped together because they contain neuraminic acid. Gangliosides have been isolated that differ in carbonhydrate composition and different types of sulfatides have been reported (see below). Eventually the sulfatides and gangliosides must be divided into the individual subclasses, and ultimately the individual molecular species in each class must be defined. The determination of the complete fatty acid (and aldehyde) composition of each lipid class is a step toward the final definition of each molecular species present in brain. The first task is to devise methods for the quantitative isolation of the broadest lipid classes and progress toward this goal is the subject of this report.

Cholesterol. Cholesterol has been determined by direct isolation as the first fraction eluted from a magnesium silicate column with total brain lipid as sample, and by separation of the first fraction from a DEAE column (eluted with chloroform/methanol 9/1) on a silicic acid-silicate column. The elution schemes are given above or have been described previously (6). Such simple methods are adequate for the quantitative isolation of pure cholesterol from beef brain because sterol esters and glycerides are not present.

TABLE VII
Composition of Whole Beef Brain Lipid

Lipid Class	% Total Lipid
Cholesterol	20.2
Ceramide	0.4
Cerebroside	16.0
Galactosylglyceride	0.5
Sulfatide	3.5
Sphingomyelin	7.8
Lecithin	10.8
Phosphatidyl Ethanolamine	16.1
Phopshatidyl Serine	7.2
Phosphatidyl Inositol	2.0
Triphosphoinositide *	0.3
Phosphatidic Acid	0.4
Cardiolipin	0.3
Ganglioside	2.6
Uncharacterized acidic lipids	2.0
Water Soluble Nonlipid	10.1
	100.2

* Includes triphosphoinositide extracted with chloroform/methanol/ammonia.

Ceramide. Ceramide has been isolated from magnesium silicate columns and by fractionation of the nonionic fraction from DEAE columns on silicic acid-silicate columns (6). The values by both methods are in good agreement.

Cerebroside and galactosylglyceride. Cerebroside has been determined by two methods. A mixture of cerebroside and sulfatide has been isolated from magnesium silicate columns and the mixture separated into cerebroside and sulfatide fractions on DEAE columns (6). The value for cerebroside by this method compares favorably with the value obtained by a combination of DEAE and silicic acid-silicate-water column chromatography (6). It is now clear that cerebroside isolated by both methods contains a small amount of galactosylglyceride. This can be degraded by mild alkaline hydrolysis without affecting the cerebroside. The cerebroside can then be separated from the fatty acids of the glyceride on a silicic acid column and the relative amounts of each lipid can thus be determined. Fatty acids are eluted with chloroform and cerebrosides with chloroform/methanol 4/1. The amount of galactosylglyceride has been determined by quantitative infrared spectroscopy using pure cerebroside to which was added phosphatidyl inositol to give the amount of ester absorption seen with brain cerebroside fractions. Values by both methods have been checked by the quantitative determination of ester groups with the hydroxamic acid reaction.

Sulfatide. Sulfatides are incompletely characterized. Nakayama (34) and Thannhauser et al. (35) presented evidence indicating that a sulfatide preparation from beef brain was cerebroside-6-sulfate. Hakomori and Ishimoda (36) described the separation of three sulfatides, and Rouser et al. (5) noted the separation of several sulfatide spots on paper chromatograms. Yamakawa et al. (37) reported that a cerebroside-3-sulfate was present in brain, and Hakomori et al. (38) separated two sulfatides from beef brain and characterized one as a cerebroside-3-sulfate and the other as a more complex sulfatide with two carbohydrate residues and two sulfate groups per lipid molecule.

Total sulfatide has been determined in this laboratory by two methods. Elution of sulfatide along with cerebroside from magnesium silicate followed by separation of the two lipid classes on DEAE was achieved

first (6). A second procedure has been developed with which sulfatide is eluted directly from a DEAE column (see above). The amounts of the individual sulfatides have not been determined.

Sphingomyelin and lecithin. These two lipids have been recovered together (along with cholesterol, ceramide, and cerebroside) from DEAE and separated on silicic acid-silicate-water columns (6). The purity of sphingomyelin is shown in Figures 6 and 7 and illustrates the performance of a silicic acid-silicate-water column for the separation of lecithin and sphingomyelin.

Phosphatidyl ethanolamine and phosphatidyl serine. Two methods have been used for quantitative isolation of these lipids. The first method utilizes a silicic acid column for the recovery of a mixture of phosphatidyl ethanolamine and phosphatidyl serine that is then separated into individual components on a silicic acid-silicate-water column (8). The separation of these two lipids has also been accomplished on DEAE columns (see 6 and above). The value for phosphatidyl ethanolamine from DEAE is slightly lower than the value obtained by the first method. This appears to be related to the presence of a naturally occurring oxidation product of phosphatidyl ethanolamine that is separated from the native form of the lipid on DEAE but not on silicic acid columns. This conclusion is based on comparisons of brain lipid with phosphatidyl ethanolamine and its oxidation products. The value determined by the DEAE method is shown in Table VII. The purity of these lipids eluted from DEAE is shown in Figure 6.

Phosphatidyl inositol. The presence of phosphatidyl inositol in brain was indicated by mild alkaline hydrolysis procedures, and Rouser et al. (6) reported the chromatographic isolation of a lipid believed to be phosphatidyl inositol. Conclusive evidence for the presence of this lipid in beef brain has now been obtained in this laboratory and an improved isolation procedure giving a more accurate estimate of the amount in whole brain lipid has been developed. We have found that phosphatidyl inositol from soybean and wheat germ are eluted from DEAE and silicic acid columns in the same manner as the lipid from brain; that the paper and thin layer chromatographic migrations for the preparations are the same; and that the preparations give similar hydrolysis products and infrared spectra. Phosphatidyl inositol has been eluted from DEAE columns along with sulfatide and the two lipids separated by elution from DEAE with different solvents as described above. The purity of the fraction is shown in Figure 6.

Triphosphoinositide. Two advances have made the estimation of triphosphoinositide possible. The total extraction of this lipid with chloroform/methanol/ammonia has been accomplished (see above) and the elution of triphosphoinositide in pure form from DEAE columns with chloroform/acetic acid 3/1 as described above has made possible the quantitative isolation of the lipid. A small amount of this lipid has been found in chloroform/methanol 2/1 extracts of brain, but most of the lipid is extracted by chloroform/methanol/ammonia.

Phosphatidic acid. The presence of phosphatidic acid (diacylglycerophosphate) in brain has now been established by quantitative isolation. Phosphatidic acid elution from DEAE is described above and is accomplished with chloroform/acetic acid 3/1 containing ammonium acetate. Phosphatidic acid is first eluted along with uncharacterized acidic lipids from DEAE, then pure phosphatidic acid is recovered from a silicic acid-silicate-water column with chloroform/methanol 4/1 containing 2% water used as the final eluting solvent. Phosphatidic acid from brain was found in this laboratory to have the same column, paper and TLC characteristics as phosphatidic acid prepared from lecithin by treatment with carrot phospholipase. The two preparations gave similar hydrolysis products and infrared spectra.

Cardiolipin. The elution characteristics of cardiolipin from DEAE columns described above have been used to isolate a fraction from brain that appears to be identical to cardiolipin of beef heart and beef heart mitochondria, although an absolutely pure cardiolipin has not been obtained from brain. The value in Table VII is an approximation that is, however, believed to be close to the actual value. The column, paper, and TLC properties of the substance from brain are identical to cardiolipin of beef heart. The migration of cardiolipin on a thin layer plate is illustrated in Figure 4 (spot 11).

Uncharacterized acidic lipids. Two uncharacterized lipid classes of beef brain are eluted from DEAE columns with chloroform/acetic acid 3/1 containing 0.001 M potassium acetate or 0.01 M ammonium acetate. These acidic substances are similar to cardiolipin in that they migrate to the solvent front on paper chromatograms with an acidic solvent (chloroform/acetone/acetic acid/water, system 15, ref. 5). One of these lipids migrates in the general region of cardiolipin on paper and TLC with several systems, but this lipid is eluted from DEAE columns along with phosphatidic acid rather than cardiolopin. The infrared spectra of these lipids are similar to that of cardiolopin, and glycerophosphate is released from both on acid hydrolysis.

The data suggest substances closely related to cardiolipin. Their elution characteristics from DEAE can be used to formulate an hypothesis regarding their chemical nature. The order of elution of acidic lipid from DEAE columns where chloroform/acetic acid 3/1 containing increasing amounts of ammonium acetate is the eluting solvent is as follows: triphosphoinositide (eluted without addition of salt), the new acidic lipids, phosphatidic acid, cardiolipin, phosphatidyl inositol, and sulfatide. The acidic lipids with the largest number of acid groups are eluted first. This suggests that the new acidic lipids are glycerophosphate polymers with at least four glycerol and three phosphate groups (cardiolipin is composed of a chain of three glycerol and two phosphate groups). Paper and TLC indicate that fatty acids would have to be present on all but one or two of the hydroxyl groups of the glycerol molecules. It is possible that the two new lipids differ from each other only in that one lipid has one free hydroxyl group and the other lipid two.

Gangliosides. Gangliosides have been isolated from cellulose columns and from DEAE columns (6). Although the cellulose column procedure is difficult to reproduce, the values obtained by the two different methods are in fair agreement. There are several types of gangliosides. This is clear from the work reported from the laboratories of R. Kuhn and E. Klenk in particular. The value in Table I includes all of the gangliosides.

Water soluble nonlipids. The nonlipid substances (carbohydrates, amino acids, salts, etc.) extracted along with lipids into chloroform/methanol 2/1 are

frequently removed by a water wash using one of the procedures described by Folch et al. (7). These nonlipids have been separated from lipids and determined in the present work by elution from DEAE columns with methanol. They have also been removed using cellulose columns (6). A very small amount of lipid that appears to be a naturally occurring oxidation product of phosphatidyl ethanolamine is also eluted from DEAE with methanol and is presently included in the value for water soluble nonlipid.

Additional problems. The quantitative isolation of the most abundant lipid classes of brain has been accomplished and some of the minor components have also been isolated quantitatively and characterized. The quantitative recovery in pure form and the characterization of the new acidic lipids referred to above, and the separation of the subclasses of sulfatide and ganglioside are outstanding problems that are rather clearly defined. Two other general problems that require further investigation are the complete separation of cerebrosides from the small amount of glycerol lipid (probably galactosylglyceride) and the isolation and characterization of some of the numerous minor components detected in a number of column fractions. The very minor components (eluted along with acidic lipids from DEAE) present a particularly difficult problem. They occur in small amounts and it is difficult to distinguish natural components from artifacts of oxidation and hydrolysis.

Results obtained by other methods. Radin and coworkers were the first to present a column chromatographic method for the isolation of cerebrosides. Crude cerebroside was isolated from magnesium silicate and separated from most other lipid by passage through a column of ion exchange resin (39). No exact comparison of values by this method and the magnesium silicate column method used in this laboratory are available, although we think that the Radin procedure will yield similar results. Long and Staples and co-workers have reported column chromatographic methods for the isolation of cerebroside, sulfatide, phosphatidyl ethanolamine and phosphatidyl serine based on chromatography on aluminum oxide (40–43). Destruction of phosphatidyl ethanolamine and phosphatidyl serine on aluminum oxide was observed. No data on the reproducibility of the procedures was presented and quantitative values were not reported. From the data available it appears that these methods are less precise than the methods discussed above. The isolation of phosphatidyl ethanolamine and phosphatidyl serine from a strong absorbent upon which degradation has been observed, as well as exposure of the lipids to air seem particularly objectionable. Renkonen has reported degradation of lecithin to lysolecithin on alumina columns (44), and Thompson et al. (45) using alumina column chromatography isolated a large amount of lysolecithin from brain lipid samples. Webster and Thompson (46) demonstrated that this earlier report was in error and reported a small amount of lysolecithin in brain, although it was not isolated and characterized. The degradation of lecithin by alumina was shown to be responsible for the earlier results. Even the small amount of lysolecithin reported by Webster and Thompson to occur in human and rat brain has not been observed by us in studies of beef brain. We observed a trace component migrating like lysolecithin in some column chromatographic fractions (6). The substance appears to be an artifact since it is not present in all beef brain lipid extracts. When isolated by column chromatography the material was clearly not lysolecithin.

TLC has been used for the study of brain lipids (47–52) and it has been suggested that TLC may be useful for quantitative determination of brain lipids. As shown by column chromatographic procedures, the complexity of the lipid pattern of brain makes it apparent that TLC will have marked limitations. There are no values reported in the literature for beef brain that can be compared with the results reported here.

Dawson (53) has reported methods for the determination of phospholipids of brain by determination of the phosphorus content of spots from paper chromatograms of the water soluble products of alkaline and acid hydrolysis of the lipids. The reported recoveries varied from 85–90% of the phosphorus of the brain lipid sample. Since more than one hydrolysis product is formed from some lipids, correction factors are necessary. Some lipids yield the same hydrolysis products, e.g. lecithin and lysolecithin, and phosphatidyl ethanolamine and lysophosphatidyl ethanolamine. A crude analysis of brain phospholipids is possible with the Dawson procedures.

Bond Types in Column Chromatography and Their General Significance

The continued development of paper and column chromatographic methods has been based upon a series of hypotheses arising from attempts to explain the chromatographic findings and these hypotheses have been used as guides for subsequent studies. Concepts of the types of bonds formed in column chromatography and how these bonds can be broken have gradually evolved to rather well established principles. The chromatographic findings have general significance, and these findings have some almost unique advantages for recognizing the types of bonds involved in the interactions of lipids with nonlipid substances. Evidently many of these bond types may be encountered in various in vitro systems and in biological systems in general.

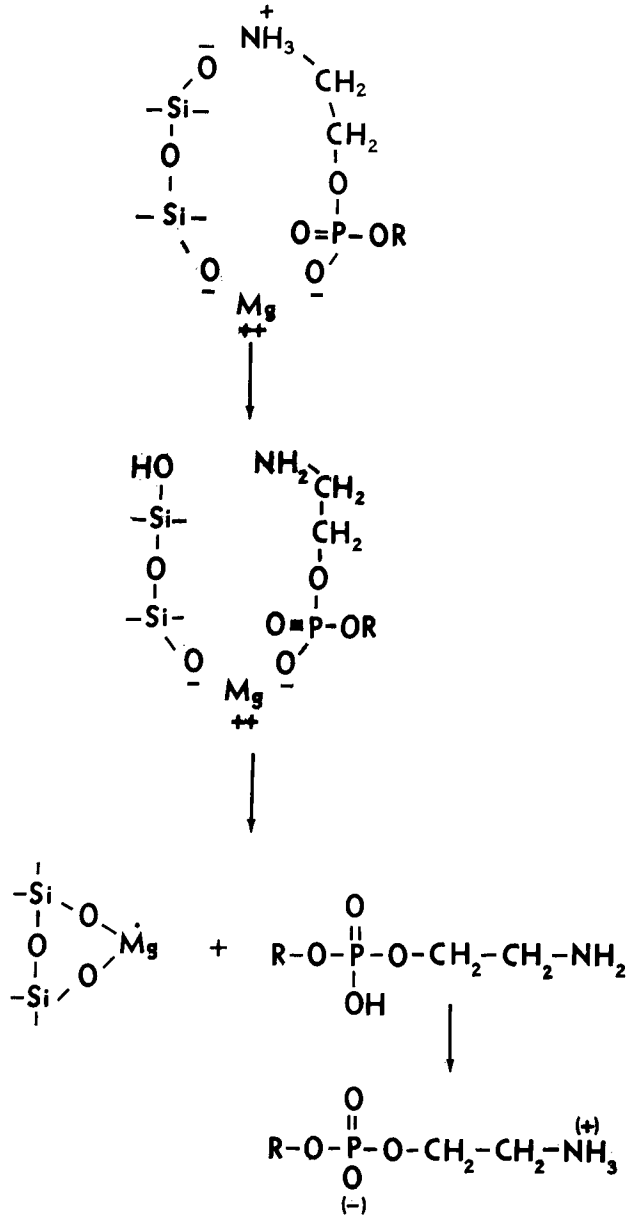
One of the most striking features of the column chromatographic method is the relative ease with which the importance of water can be demonstrated. It is well known that natural lipoproteins contain water, but the way this water is bound has remained uncertain. The results from column chromatography may be useful in the formulation of lipoprotein structures.

The following discussion of bond types should be of interest to the lipid chemist utilizing chromatographic procedures since these bond types help to explain the mechanisms underlying the separations. They should also be of interest to the biochemist in his analysis of the structures of complex biological systems and in vitro model systems.

Silicic Acid. The interaction of lipids with silicic acid has been discussed previously (6). The two basic forms of interaction are hydrogen bonding and electrostatic (coulombic) bonding, the latter being frequently mediated by ion exchange (proton transfer). Hydrogen bonding is through the Si = O, Si − O−Si, and Si−OH groups of silicic acid while the ion exchange reactions occur by proton transfer of the Si−OH group with proton acceptor groups (phosphate, sulfate, and amino) of the ionic lipids. The two forms of interaction may be combined in one molecule.

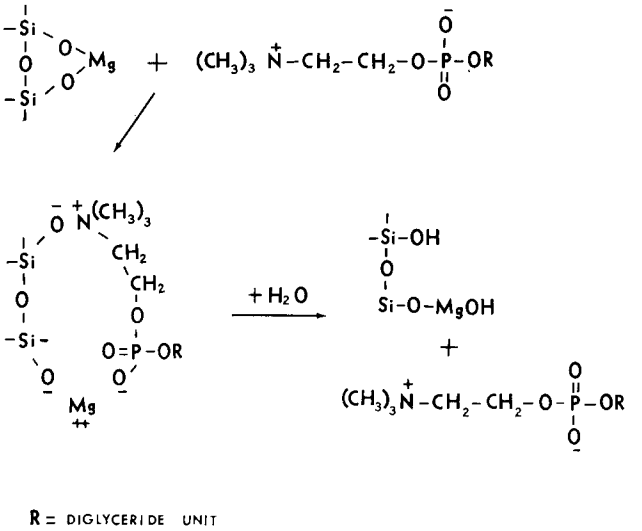
Silicates. Several interesting bonding types occur with silicates that are quite distinct from those with silicic acid. Silicates are different from silicic acid in that they are proton acceptors rather than proton donors. Silicates of monovalent and divalent ions can form different types of bonds. We have previously presented a postulate to explain the extremely strong bonding of the acidic lipid phosphatidyl serine (as well as other acidic lipids) to silicate by interaction of the acidic lipid through water to the silicate ion (6). The interaction through water can be depicted as hydrogen bonding through the carbonyl group of a carboxylate ion and Si=O or Si—O— groups or between the hydrated cations of both the acidic lipids and the cation (sodium, potassium, or ammonium) of the silicate.

One of the most significant types of interaction that has been disclosed by column chromatographic studies is that involving silicates of divalent ions with various lipids. Here direct binding to the column through acid groups can take place. Two general types of interactions can be visualized. Phosphatidyl ethanolamine can interact with magnesium silicate to give a chelate type structure that is held together by ionic forces.



This is a surprisingly strong form of interaction in the absence of water. When a magnesium silicate chromatographic system is maintained completely free of water (by heat activation of the adsorbent and dehydration of solvents with molecular sieve 5A), chloroform/methanol mixtures are poor eluting solvents for phosphatidyl ethanolamine and methanol alone elutes this lipid rather slowly (6). By the ion exchange mechanism for the dissociation of phosphatidyl ethanolamine from magnesium silicate, the transfer of a proton from the $-NH_3^+$ group can be to the silicate ion or to the phosphate group. In either case magnesium silicate is regenerated and phosphatidyl ethanolamine may escape from the binding site on the adsorbent.

The reversible reactions shown for phosphatidyl ethanolamine are not possible, however, with substances such as lecithin or sphingomyelin.

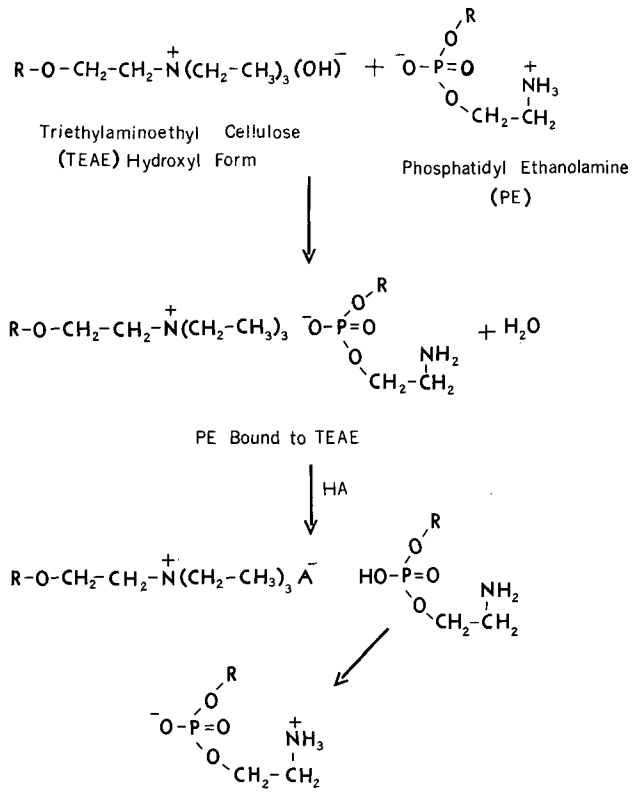


The quaternary ammonium group can not engage in proton transfer reactions and the chelate type structure held together by strong ionic bonds is not broken with absolute methanol in the absence of water. Chloroform/methanol/water mixtures elute these lipids slowly with formation of a hydrated magnesium silicate (elution of lecithin and sphingomyelin with chloroform/methanol mixtures containing even traces of water is detectible). This is in marked contrast to the behavior of phosphatidyl ethanolamine which is eluted with absolute methanol (no water required). The bonding type just considered for lecithin and sphingomyelin is of interest for biological systems since divalent ions (calcium and magnesium) present in tissues may help to bind lipids to nonlipid materials (proteins and nucleic acids) in a similar manner. After lecithin or sphingomyelin are bound to magnesium silicate, the column may be treated with water alone without elution of any appreciable amount of lipid. Despite the necessity for water in the eluting solvent, water alone can not detach lipid. Water alone does not appear to penetrate through the relatively nonpolar barrier of fatty acid chains into the area of binding of lipid to nonlipid. Evidently an appropriate organic solvent (chloroform/methanol or methanol) must be used with water. The role of the organic solvent can be visualized as aiding the penetration of water through the nonpolar lipid layer to reach the polar ionic sites. If magnesium silicate is prepared in water and an aqueous dispersion of lipid

is passed through the bed, lipid is not bound to any appreciable extent. This behavior is readily understood and explained, but it is important to note that water can prevent the formation of certain types of bonds that, once formed, will be stable in its presence.

DEAE Cellulose. The previous description of the method of interaction of lipids with diethylaminoethyl cellulose (6) can be supplemented to a certain extent. Zwitterion lipids such as lecithin and sphingomyelin show very little tendency to bind to DEAE cellulose and a freely reversible ion exchange reaction with the adsorbent can be visualized. Phosphatidyl ethanolamine is a proton donor in the zwitterion form and is retained more firmly by DEAE. The retention of phosphatidyl ethanolamine is great enough to make DEAE useful for recovery of this lipid in pure form as described above. The validity of the ion exchange reactions formulated as responsible for this separation is substantiated by studies with other types of cellulose.

Phosphatidyl ethanolamine is not retained from chloroform/methanol 9/1 to any appreciable extent by cellulose without ion exchange groups in contrast to the retention on ion exchange celluloses. Evidently the ion exchange groups of the ion exchange celluloses do function to bind the lipid. Phosphatidyl ethanolamine is bound to triethylaminoethyl cellulose (TEAE) and is not eluted with neutral solvents as it is from DEAE. This interaction can be depicted as:



The bonding to triethylaminoethyl cellulose is not reversible in a nonionic solvent since TEAE is not a proton donor. The elution of phosphatidyl ethanolamine from TEAE is accomplished with either acidic or basic solvents.

Another observation in agreement with the ion exchange concepts for phosphatidyl ethanolamine interaction with DEAE is the behavior of ceramide aminoethylphosphonate. This new lipid with amino and phosphonate groups emerges from a DEAE cellulose column along with phosphatidyl ethanolamine.

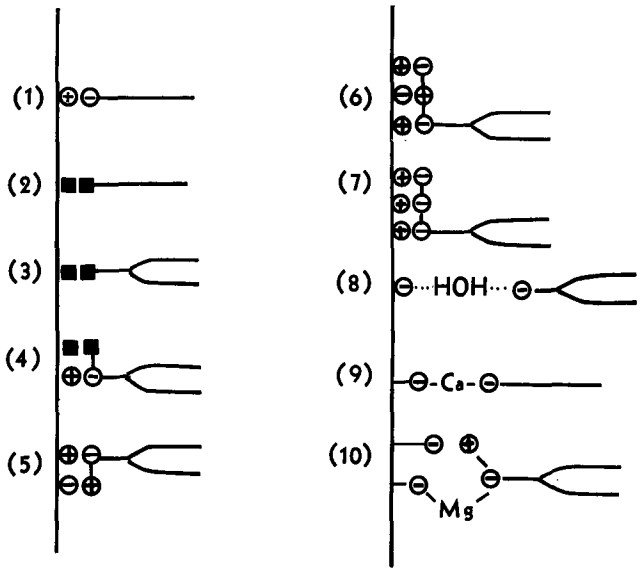


FIG. 48. Schematic representation of bonding types encountered in column chromatography. ⊕ and ⊖ indicate positive and negative charges while the squares represent polar nonionic (usually hydroxyl) groups. See text for comments.

Summary of Bond Types. Bonding types that have been encountered in column chromatographic work that may be important in biological systems are shown in Figure 48. Bonding type 1 is a simple interaction of positively and negatively charged groups encountered when the salt of a fatty acid is bound to DEAE. The bond is broken with acidic or basic solvents or by inclusion of salt in an eluting solvent.

Bond types 2 and 3 are the simplest forms of a polar nonionic interaction (hydrogen bonding) as for cholesterol, cerebroside, and galactosylglycerides. Bonding type 4 is a combination of interaction by ionic groups and nonionic polar (hydroxyl) groups that can be visualized for phosphatidyl inositol interacting with DEAE cellulose. Bonding type 5 is an interaction typical of lecithin and sphingomyelin and is labile. The adsorption site and the molecule being adsorbed are internally balanced and may dissociate in the absence of an ionic solvent. Bonding type 6 is possible with phosphatidyl serine, while bonding type 7 can be visualized for a more complex lipid such as the triphosphoinositide interacting with ion exchange sites of DEAE celluloses. This is a simplification of the triphosphoinositide structure since two of the phosphate groups on the inositol have two negatively charged groups under biological conditions. Bonding type 8 is an illustration of bonding of two anionic substances through water. This is believed to take place when phosphatidyl serine is bound to a silicate of a monovalent ion and the mode of interaction has been used to explain the separation of phosphatidyl serine from other lipids (6). Bonding type 9 is typical for magnesium or calcium silicates and can be formed from the salt of a fatty acid with a silicate of a divalent ion. Bonding type 10 is the chelate type structure discussed above that can be formed with lecithin or sphingomyelin and magnesium silicate.

A bimolecular lipid structure of the type thought to be important for biological membranes can be formulated using the various types of ionic and hydrogen bonding interactions discussed above. The bonds can be considered as joining the lipid-nonlipid layers

and perhaps some of the lipid molecules to each other.

It seems probable that studies of model systems based upon the above observations will be profitable. It is apparent that various chromatographic adsorbents can be replaced by proteins or other substances of biological interest and the binding of lipid under various conditions can be studied. The binding of lipid to an insoluble protein can be studied in chloroform or a hydrocarbon solvent in which the protein is insoluble. The role of water, the number of molecules bound per mole of protein, and other factors affecting binding and bond breakage can be studied. The substances can be brought together in a test tube and the bonding studied by separation of the insoluble portion by centrifugation, although some substances may be solubilized by the lipid. A detailed study of such interactions will undoubtedly aid appreciably in formulating lipoprotein structures.

General Conclusions

Lipid chemists can look forward to a very bright future. With the great increase in knowledge of lipids and the greatly improved methods for their isolation and characterization, some of the important biological problems concerning the methabolism and functions of lipids can now be approached with confidence. The solutions to many important problems now seem only a matter of time.

It is evident that the job of the lipid chemist has only begun. There are many new lipid classes to be isolated and characterized, and each lipid class must be determined quantitatively. The amount of each lipid class and the precise fatty acid composition must then be determined under various physiological conditions and in various pathological states. Ultimately each lipid class will be separated into the individual molecular species. Several ways that individual molecular species can be separated are known and these will be explored. The separation of appropriate derivatives by gas-liquid chromatography and the separation of the mercury adducts will without doubt find broad application for this purpose. When individual molecular species can be recovered, isotope studies can establish relative turnover rates and focus attention on important differences within a lipid class.

When the skills of the lipid chemist are applied to the problems of lipid composition of subcellular particles, biological membranes, and specific lipoproteins, a better understanding of the relationships between lipid structure and biological function will be obtained and important metabolic pathways can be studied in even greater detail.

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