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Review

Chromatographic separation, identification, and analysis of phosphatides*

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The advances in lipid methodology over the past decade have been remarkable and have served to expedite research in this field. The rapidity of progress makes it necessary, from time to time, to review the latest techniques in sufficient detail to demonstrate their practical application to lipid problems. Such a review must necessarily be limited in scope and the author regrets that it will not be possible to discuss all the valuable papers in the area. The present review concerns recent developments in the use of chromatography for the separation, identification, and analysis of the intact phosphatides. Analytical procedures for the component parts of the phosphatides, namely fatty acids, aldehydes, alcohols, nitrogenous bases, nitrogen, and phosphorus will be covered briefly. Discussion will be restricted to phosphatides found primarily in mammalian tissues and emphasis will be placed on micromethods rather than on preparative procedures. Texts (1 to 6) and reviews (7 to 12), covering the chemistry, preparation, and analysis of the phosphatides on a more macro level are available.

LIPID EXTRACTION

The primary problem is the isolation of the lipids from their biological source. There is no single standard method for lipid extraction; the method depends on the nature of the problem and the type of biological material under study. Thus, the extraction of lipids from serum presents a relatively simple problem compared to the extraction of lipids from tissues, bacteria, or plants. These materials usually require some type of fragmentation, such as sonic disintegration, homogenization, mechanical grinding, or compression-decompression. Lyophilization may be desirable before fragmentation and extraction. During these steps, it is important to keep the chemical, physical, and enzymatic degradation of the lipids to a minimum. This is usually accomplished by the control of temperature, chemical environment, and time of exposure of the material to each treatment. When working with phosphatides, the use of a nitrogen atmosphere and moderate temperatures is recommended. Prolonged Soxhlet extraction, for example, can lead to extensive lipid degradation.

The solvents most commonly employed for lipid extraction, and to which this discussion will be limited, are methanol-ethyl ether or methanol-chloroform. Once the extract is made, it must usually be rectified to remove non-lipid material, such as amino acids, salts, urea, sugars, and water-soluble phosphate esters. Various washing procedures (13 to 18) have been used, the more common ones employing water alone or aqueous salt solutions. The washing of the lipid extract with aqueous solutions must be done with great care. Some lipids, such as lysolecithin, are appreciably soluble, both in water and in organic solvents, and their loss must be avoided.

Phosphatides also promote the formation of stable emulsions, which may lead to loss of lipid. A particular problem with phosphatides is their tendency to solubilize non-lipid material in organic solvents (19). Complexes of phosphatides with salts also occur and these may not be easily dissociated. These factors must be considered even when the washing is performed under the gentle conditions such as described by Folch (14).

Removal from a lipid extract of water-soluble radioactively tagged substances (such as choline or glycerol) is important even though they may occur in such small amounts as to escape detection by ordinary

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analysis. It may be necessary to compromise in removing water-soluble contaminants, as the washing procedure must be extensive and is likely to result in loss of some lipid material.

The removal of these contaminants by other methods has been suggested. Effective removal of non-lipid material from a lipid extract has been reported using dialysis (20), electrodialysis (21), electrophoresis (22), paper chromatography (23), and column chromatography (24). It has also been done by spotting on paper and then washing the paper (25).

The lipid extract may not necessarily require washing. It is customary in some laboratories to evaporate the first extracts to dryness under nitrogen *in vacuo* by the use of rotary evaporators. The resulting residue is then re-extracted with chloroform, petroleum ether, or ethyl ether. This procedure effects a partial rectification of the original extract, but some non-lipid contaminants are usually present. If paper chromatography is used to separate the phosphatides, such contaminants do not interfere (26, 27) since they remain at or near the origin on the chromatograms. This is true for GP, P_i, GPC, GPE, GPS, GPI, ATP, CTP, and amino acids.[1] The water-soluble materials may then be removed by washing the chromatogram with water, the phosphatides remaining bound to the paper.

Degradation of the phosphatides must be minimized in order to permit the most effective use of paper and column chromatography. Peroxidized lipids do not chromatograph as discrete spots or bands, and degraded lipids give rise to artifacts.

ANALYTICAL METHODS FOR THE HYDROLYSIS PRODUCTS OF PHOSPHATIDES

This section briefly covers the chemical methods commonly used for the analysis of the component parts of the phosphatides. All the methods are not included as they are described in detail elsewhere (1, 2, 3, 5). Inasmuch as the complete identification of a phosphatide requires that all its component parts be known, the procedures for the analysis of each of these components are specifically included.

Lipid Phosphorus. Phosphorus determination can be done by a number of procedures (28 to 35). In our experience, the method of Harris and Popat (34) has been excellent in the 10- to 40-μg range, and a modified procedure of Bartlett (32) has given good results in the 0.5- to 5.0-μg range. The modified Bartlett procedure utilizes perchloric acid in place of sulfuric acid and is described later in this review.

Lipid Nitrogen. Nitrogen analysis presents certain difficulties as choline-N may not be quantitatively converted to ammonia by some methods. The Kjeldhal procedure or the use of Nessler's reagent will give low values; these two procedures are excellent for amino-N, such as occurs in PE, PS, or sphingosine. The quantitative analysis of choline-N (lecithin, sphingomyelin) is best done by the Dumas procedure.

Lipid Ester. Ester groups can be determined by the use of the hydroxamate-ferric chloride reaction (36 to 39). The methods of Rapport and Alonzo (37) and of Snyder and Stephens (38) have worked well in our experience.

Lipid Aldehyde. Plasmalogens are hydrolyzed with acetic acid-mercuric chloride. The liberated aldehydes are then analyzed either with the p-nitrophenylhydrazine method (40) or with the Schiff reagent (41 to 43). With both procedures it is difficult to obtain good reproducibility and high precision.

Analysis for Nitrogenous Bases. Various methods are available for the analysis of choline (44 to 51), serine and ethanolamine (52 to 54), and sphingosine (55). The analysis of N-methylethanolamine and N,N-dimethylethanolamine, however, is difficult to perform and has not been worked out on a routine basis.

Lipid Amino Groups. Lea and Rhodes (56) have published a method for the direct analysis of amino-phosphatides by the use of the ninhydrin reagent. The method involves certain assumptions and may not be very precise.

Lipid Glycerol and Inositol. The analyses of glycerol (57, 58) and inositol (12, 59 to 61) require more laborious chemical or microbiological assays. The phosphatides must be completely hydrolyzed to free glycerol and to free inositol in order for these methods to be quantitative.

PAPER CHROMATOGRAPHIC IDENTIFICATION OF HYDROLYSIS PRODUCTS OF PHOSPHATIDES

Glycerol and inositol and their water-soluble phosphate esters can be separated and identified on paper as described by Eberhardt and Kates (62), Grado and Ballou (63), Dawson (64), and Maruo and Benson (65). Serine, ethanolamine, and choline are separated and identified by various procedures (52, 53, 62, 63, 66, 67). Sphingosine and sphingosine phosphates are more difficult to chromatograph but can be separated by the methods of Dawson (68) and Karlsson (69).

ANALYSIS OF PHOSPHATIDE FATTY ACIDS, ALDEHYDES, AND SPHINGOSINE BY PAPER CHROMATOGRAPHY AND GAS-LIQUID CHROMATOGRAPHY

The fatty acid components of the phosphatides can be identified by paper chromatography in a number of

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[1] The following symbols are used to designate the water-soluble phosphate esters: GP, glycerylphosphate; GPC, glycerylphosphorylcholine; GPE, glycerylphosphorylethanolamine; GPS, glycerylphosphorylserine; GPI, glycerylphosphorylinositol; P_i, inorganic orthophosphate; GPG, diglycerylphosphate; GPGPG, triglyceryldiphosphate; DMGPE, N,N-dimethylglycerylphosphorylethanolamine; MMGPE, N-monomethylglycerylphosphorylethanolamine; GPIP, glycerylphosphorylinositolphosphate; $GPIP_2$, glycerylphosphorylinositoldiphosphate. The following symbols are used to designate the various intact phosphatides: LEC, lecithin; PE, phosphatidyl ethanolamine; PS, phosphatidylserine; PGP, polyglycerylphosphatide (cardiolipin); SPH, sphingomyelin; lyso-LEC, lysolecithin; lyso-PE, lysophosphatidylethanolamine; lyso-PS, lysophosphatidylserine; MPI, monophosphoinositide; DPI, diphosphoinositide; TPI, triphosphoinositide; plas, plasmalogen; PA, phosphatidic acid. Other symbols used: Et, ethanolamine; Ch, choline; Ser, serine; TCA, trichloracetic acid.

systems (70 to 80). In our experience, the procedures of Kaufmann and collaborators have proved to be effective (70, 73, 74, 75). Fatty acid derivatives have also been separated on paper. Thus, the mercuric acetate derivatives (81), the 2,4-dinitrophenylhydrazides (82), the 2,4-dinitrophenylhydrazones of the p-bromphenacyl esters (83, 84), and the chlorphenacyl derivatives (80) have been separated on paper in a very effective manner.

The use of gas-liquid chromatography for the analysis of the fatty acids is becoming a routine procedure in many laboratories (85 to 91). Fatty aldehydes (92) and sphingosines (93) have also been separated by this versatile technique. Although gas-liquid chromatography is a very useful technique, it is by no means infallible. Identification by this means can only be made if the conditions of the analysis are carefully controlled and if suitable standards are analyzed. Gas-liquid chromatography suffers from the same limitations as paper and column chromatography; in some cases it is more limited, as the isolation of a given component is often very difficult and additional tests cannot be made. The reliability of the identification will depend on the complexity of the mixture and, particularly in the case of minor components, on the availability of supporting chemical, spectral, and physical data.

PAPER CHROMATOGRAPHY OF THE PHOSPHATIDES

As it is our major aim to discuss and evaluate paper chromatographic methods for the identification of the phosphatides, the remainder of this review deals with the various methods reported in the literature and covers in detail those methods that are most promising and that have found wide application.

Intact Phosphatides. The intact phosphatides can be chromatographed directly without resorting to hydrolysis. Chromatography may be carried out on filter paper (94 to 104), formalin-treated filter paper (105 to 108), acetylated paper (109), tetralin-impregnated paper (110), silicic acid-impregnated paper (111 to 115), silicic acid-impregnated glass fiber paper (116, 117, 118), phosphate-impregnated paper (114), and on a thin layer of silica gel coated on a glass plate (119). Each of these methods has its own inherent advantages, disadvantages, and limitations. Consequently, the selection of a particular method will depend on the nature of the research problem and on the skills and inclinations of the investigator. Silicic acid-impregnated filter paper or silicic acid-impregnated glass fiber paper have been most widely used (120 to 138); their use will be discussed in greater detail as they allow for quantitative analysis of the phosphatides.

1. *Non-impregnated Filter Paper.* Chromatography of the phosphatides on non-impregnated filter paper was first attempted during the period 1952–1958 by several laboratories throughout the world (94 to 103). The solvent systems developed were only partially successful and were applicable only to rather simple mixtures. Acylation of the amino-lipids prior to chromatography allowed the use of more material and effected a separation of the phosphatidylcholines from the N-acyl cephalins (100). The use of circular disk chromatography gave some separations on a microscale but was not suitable for quantitative work (97, 103). Solvents containing ketones and acetic acid proved to be reasonably effective (102, 103), but their use was also limited because of the small capacity of the systems, creating difficulties in detection and identification. Wide application of these systems was therefore not realized.

2. *Formalin-Treated Paper.* Hörhammer *et al.* (105, 106, 107) developed chromatographic procedures using formalin-treated paper. The solvent system consists of butanol-acetic acid-water, 4:1:5 (v/v). The separations reported by these workers appear to be fairly good for some phosphatides but the resolving capacity is not so great as that obtainable on silicic acid-impregnated paper. The systems of Hörhammer are useful for the separation of the inositol phosphatides and PS. However, PE and LEC are not resolved. Three types of diphosphoinositides are reported to be separated by this method.

3. *Tetralin-Impregnated Paper.* Inouye and Noda first succeeded in separating individual lecithins by paper chromatography on tetralin-impregnated paper (110). The separation was achieved by converting the lecithins of egg yolk to their mercuric acetate addition compounds. The lecithins were resolved into five components but there was appreciable overlapping between some of the components. The unsaturated lecithins moved faster than the more saturated ones. This work represents a start toward the resolution of a difficult problem, one which must eventually be conquered if the metabolic activity of phosphatides having different fatty acids is to be revealed. It is well to keep in mind that the various phosphatides observed on chromatograms represent families of compounds varying with respect to their fatty acid composition.

4. *Thin-Layer Glass Chromatography.* Recently Wagner and co-workers (119) used thin-layer chromatography for phosphatide separations. Silica gel was coated on a glass plate to form the stationary phase. The solvent system used consisted of chloroform-methanol-water, 65:25:4 (v/v). The chromatograms

appeared to be very good. The method certainly has its place as a technique for phosphatide separation but suffers from some disadvantages. It is less versatile and requires more expensive apparatus than the usual procedures utilizing paper. The major advantages of this method are that it is possible to use more material and to use certain detection tests not possible on paper (e.g., charring).

5. *Silicic Acid-Impregnated Filter Paper.* Very useful separations of the phosphatides are achieved by chromatography on silicic acid-impregnated paper. The chloroform-methanol system of Lea, Rhodes, and Stoll (111), the diisobutyl ketone-acetic acid-water systems of Marinetti *et al.* (112, 113), and the chloroform-methanol-ammonia system of Rouser *et al.* (114) have given excellent separations and have been widely used. The system of Marinetti *et al.* is more effective in separating a complex phosphatide mixture as obtained in a total lipid extract of a tissue and has found widespread use (122 to 137) in a variety of problems. The method is described below in detail, both the technical aspects of preparing the papers and the detection tests and quantitative analyses. Typical chromatograms and autoradiograms obtained by this method are shown in Figures 1, 2, 3, 6, and 7.

a) Reagents and Equipment Required for Impregnating the Papers.

(1) *Whatman No. 1 filter paper.* Cut into sheets 21 x 20 cm (paper A) and 12 x 42 cm (paper B). Draw a pencil line 3 cm from the bottom edge of paper A (so as to run parallel with the 21-cm edge) and 6 cm from the bottom of paper B (so as to run parallel with the 12-cm edge).

(2) *Sodium silicate solution.*[2] Dissolve 295 g of NaOH in 500 ml of distilled water. In a 2-liter beaker, suspend 310 g of silicic acid (Mallinckrodt, analytical reagent, 100 mesh for chromatography) in 800 ml of distilled water. Add the NaOH solution to the silicic acid slurry with stirring, allow to cool, and dilute to 1,700 ml with water. This solution is used for impregnating the papers.

(3) *4N HCl* (2 to 4 liters).

(4) *Pyrex trays* (8 x 12 in. and 11 x 17 in.).

(5) *Rhodamine 6G. Stock solution:* Dissolve 240 mg of Rhodamine 6G (C.I. 752) (National Aniline Division, Allied Chemical and Dye Corp., 40 Rector St., New York, New York) in 1 liter of distilled water and let stand overnight. *Working solution:* Dilute 50 ml of the stock solution to 1 liter with distilled water.

(6) *Chambers and solvents.*[3] For papers A, use 2-quart special wide-mouth Mason jars or other suitable containers (lids are protected with aluminum foil or Teflon). The solvent system is diisobutyl ketone–acetic acid–water, 40:20:3 (v/v) (use 50 ml). This system requires about $3^{1}/_{2}$ to $4^{1}/_{2}$ hours to run (Figs. 1, 2, 4, 5).[4]

For papers B, use Pyrex cylinders 6 inches i.d. and 18 inches in height. The solvent system is diisobutyl ketone–acetic acid–water, 40:25:5 (v/v) (use 200 ml). The system requires 16 to 20 hours to run. The 12- x 42-cm paper can be cut into three 4- x 42-cm strips and chromatography can be carried out in 1-liter graduate cylinders having glass-stoppered necks. Chromatography is carried out at a temperature of 23° (a constant temperature room is desirable).

b) Impregnation Procedure. The sheets of Whatman paper (A or B) are carefully dipped only once into the sodium silicate solution (use the 8- x 12-in. Pyrex tray) until the paper is just wet. It has been found best to dip one edge (either the 21- or 12-cm edge, respectively) into the silicate solution in such a way that a straight front moves up the paper to within $^{1}/_{2}$ inch from the top. The paper by this time has been immersed in the silicate solution for 5 to 10 seconds. Remove the paper and carefully hang 1 to 2 minutes to allow excess reagent to drip off. The drippings that accumulate at the lower edge of the paper are carefully removed by means of a glass plate.

After they have been allowed to hang for 1 to 2 minutes, the papers are immersed in the 4N HCl bath (use the 11- x 17-in. tray). It is convenient to prepare 12 to 20 papers at one time. When successive papers are to be placed in the HCl solution, it is necessary to avoid contact between the newly dipped paper and the papers in the bath until the silicate on the paper has had a chance to react with the HCl. This is accomplished by laying the newly dipped paper on the surface of the HCl momentarily and picking it up, repeating this manipulation twice. When the paper has had a chance to react with the HCl (usually within 10 to 15 seconds), it can be immersed in the bath with the other papers. The papers are allowed to stay in the bath for 15 minutes, after which the HCl is decanted off and distilled water added. This washing procedure is repeated six times, allowing 10 minutes for each wash. The papers are then hung in a hood to dry, and are pressed between glass plates overnight. The next day the papers are ready for use. The papers can be safely stored for months. A closed storage chamber is recommended to avoid adsorption of vapors.

c) Applying the Lipids to the Paper. Paper A: Divide the paper into eight 2.5-cm sections or ten 2-cm sections along the 3-cm line. The phosphatides are applied in volumes of 10 to 30 μl at the 3-cm starting line. For a total lipid extract, the amount of P per spot should be between 0.5 and 2.0 μg (12 to 50 μg of total phosphatides). For individual phosphatides, the amount of P per spot should be between 0.2 and 0.5 μg (5 to 12 μg of phosphatide).

Paper B: Divide the paper into four 3-cm sections along the 6-cm line. The phosphatides are applied in volumes of 20 or 30 μl at the starting line. For a total lipid extract from tissues, the amount of P per spot should be 6 to 8 μg (150 to 200 μg of phosphatides). Above 8 μg per spot, the lipid components begin to elongate and overlapping may result. For individual lipids the amount of P per spot should be 1 to 2 μg (25 to 50 μg of phosphatide).

d) Development. Ascending chromatography is recommended. The 21- x 20-cm papers (paper A) are rolled into cylinders and the ends held together by stainless steel wire. The vertical ends of the paper must not touch each other. The rolled cylinder is then placed in a Mason jar, the chamber sealed, and the chromatogram developed. This requires $3^{1}/_{2}$ to

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[2] The addition of 0.1% soluble starch to the sodium silicate solution has been found to eliminate most of the powdery nature of impregnated papers and still permits good resolution of the phosphatides.

[3] For accurate and reliable results, all glassware must be thoroughly acid-washed. The chromatograms should be extracted on the same day they are developed if they are to be used for quantitative work; phosphatides are not quantitatively extracted from chromatograms allowed to stand for more than 1 or 2 days.

[4] The substitution of isotonic sodium chloride for water in the diisobutyl ketone-acetic acid-water solvents has been found to improve these chromatographic systems by allowing for better and more reproducible chromatograms. In particular the phosphatides are more discrete and the separation of lysolecithin from inositol phosphatide and of phosphatidyl serine from lecithin is improved.

$4^1/_2$ hours. Typical chromatograms are shown in Figures 1, 2, 4, and 5.

The 12- x 42-cm papers (paper B) are suspended from a suitable support and allowed to dip into the solvent. The chamber is sealed and the chromatogram allowed to develop for 16 to 20 hours. The 12- x 42-cm paper may be cut into three 4- x 42-cm strips and chromatography carried out in 1-liter graduate cylinders. This requires 16 to 20 hours. An autoradiogram of such a run is shown in Figure 3.

DETECTION AND IDENTIFICATION OF THE PHOSPHATIDES ON PAPER CHROMATOGRAMS

The mobility and spot tests provide valuable information for the identification of the chromatographic components. The identification is usually fairly reliable but may not be unequivocal, depending upon the nature of the lipid being studied. In most cases, other supporting evidence should be obtained.

Rhodamine 6G Test for All Phosphatides. The paper chromatograms are dried in a hood for 1 hour and then immersed for 2 to 3 minutes in the Rhodamine 6G solution. The excess dye is rinsed off with distilled water and the wet chromatograms are viewed under ultraviolet light (366 mμ). The phosphatide spots appear yellow, orange, purple, or blue (113). The acidic phosphatides usually stain blue or purple whereas the neutral phosphatides stain yellow. On dry chromatograms, all the lipids appear yellow. A photograph of a Rhodamine-stained chromatogram as seen under ultraviolet light is given in Figure 1.

Ninhydrin Test for Amino-Phosphatides. The dry chromatograms are sprayed or dipped once into a 0.25% solution of ninhydrin in acetone-lutidine, 9:1 (v/v). Lutidine must be used or the color reaction is very weak. The papers are allowed to stand for several hours at room temperature; heating the paper should be avoided. The amino-lipids appear as purple spots. The test is quite specific for phosphatides having a free amino group.

Permanganate Test for Unsaturation. The dry chromatograms are rinsed with distilled water for 10 minutes and then immersed in a 1% aqueous $KMnO_4$ solution for about 1 minute. The excess $KMnO_4$ is removed by washing with distilled water. Unsaturated compounds appear as brown spots.

Iodine Vapor Test for Unsaturation. Some crystals of iodine are placed in the bottom of an appropriate sized jar; the jar is covered and placed in a pan of hot water (about 60°). When the inside of the jar is filled with purple iodine vapors, the dry chromatograms are introduced for about 30 seconds to 1 minute. They are then removed and observed in daylight and under ultraviolet light (366 mμ). In daylight the positive spots appear brown; under ultraviolet light these brown spots appear intensely dark. The color fades on standing. Exposure to osmium oxide vapors can also be used to detect unsaturated lipids (97).

Test for Choline. The dry chromatograms are washed with distilled water for 10 minutes and then immersed in a 1% aqueous solution of phosphomolybdic acid for 10 minutes. The papers are washed three times for 10 minutes each in distilled water and then immersed in a 1% solution of $SnCl_2$ in 3N HCl. Spots containing choline appear blue (139). This test is not specific for choline since weaker tests are given by unsaturated cephalins and other unsaturated lipids. The Dragendorf reagent can also be used to test for choline (140). The choline lipids give a pink or reddish-pink color with this reagent.

Test for Plasmalogen. The dry chromatograms are washed in distilled water three times for 10 minutes each time and then dried. The chromatograms are then immersed for about 1 to 2 minutes in a solution of 2,4-dinitrophenylhydrazine (150 mg dissolved in 100 cc of 3N HCl). The chromatograms are washed in distilled water four times for 10 minutes each time to remove excess reagent. The wet or dry chromatograms are viewed under ultraviolet light. A test is only considered positive if the spots absorb in the 366-mμ

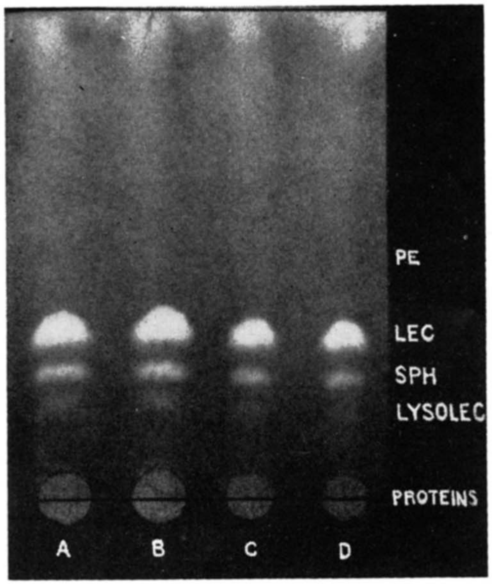

FIG. 1. Photograph of a chromatogram of human serum phosphatides. The photograph was taken of a dry chromatogram stained with Rhodamine 6G. The light source was a Mineralight ultraviolet lamp, 257 mμ. A and B represent 30 μl of serum; C and D represent 20 μl of serum. The serum was applied directly to the paper (206). Chromatography was done on the 21- x 20-cm papers in Mason jars. The solvent system was diisobutyl ketone–acetic acid–water, 40:20:3 (v/v). The temperature of the run was 23°. The time of the run was about 4 hours. The spots at the origin represent the serum proteins. MPI may occur with lyso-LEC.

region; they will then appear as dark areas. In some cases, the spots appear orange in visible light but do not absorb in the ultraviolet. This is caused by adsorption of the reagent and does not constitute a positive test for aldehyde. The Schiff test on chromatograms is less specific and hence has limited application in confirming the presence of plasmalogens; it has been used by Hack (97, 129, 141) and by Rouser *et al.* (114) and, under controlled conditions, may be reliable and useful.

Tricomplex Staining. Hooghwinkel *et al.* (135, 142) have developed a procedure in which phosphatides are made to complex with uranyl ions and acid fuchsin. The reaction is pH dependent. The triple complex formed can also be coupled with brilliant green or with Rhodamine 6G. The tricomplex staining reaction occurs with phosphatides of the acid-base type (lecithin, PE, sphingomyelin). The combined tricomplex-brilliant green test permits the detection of the acid-base and acidic lipids (such as MPI, DPI, and PGP). The combined tricomplex-Rhodamine test allows for the detection of all the lipids.

Reaction for Primary Amines. Hooghwinkel *et al.* (135) have reported a test for phosphatides containing a primary amine group. The amines react with CS_2 in the presence of triethylamine to yield dithiocarbamates. The dithiocarbamates are then decomposed with acidified $AgNO_3$ to yield Ag_2S.

Test for Phosphate. The Hanes and Isherwood test (143) allows the detection of phosphatides on chromatograms. There are several modifications of this test depending on how the formation of the phosphomolybdenum blue complex is enhanced. Hydrogen sulfide, stannous chloride, ferrous sulfate, vitamin C, and ultraviolet radiation have all been reported to expedite the formation of the blue complex. The background color must be minimized in order to obtain good contrast of the spots. Hooghwinkel *et al.* (135) have reported a different technique to detect phosphate. They immerse the chromatogram in a diluted solution described by Zinzadze and state that the blue spots develop within a few minutes (135).

Protoporphyrin Test for Lipids. The detection of lipids by staining with protoporphyrin has recently been published (144). The authors claim this test to be very sensitive and superior to staining with Rhodamine 6G. In our experience, however, the protoporphyrin test has been less sensitive than the Rhodamine. This serves to point out the variation that can be anticipated by spot tests in the hands of different workers.

Charring Test. The detection of lipids by spraying chromatograms with sulfuric acid-water, 1:1 (v/v) and heating has been reported to be an effective method for chromatograms run on glass fiber paper (116, 117, 121) or on glass plates coated with silica gel (119). The lipids char and yield brownish-black spots.

Other Dye Tests. Biebrich scarlet (97), Nile blue (97), Rhodamine B (101, 119), and fast acid violet (101) have been used for lipid detection but have not found wide application.

Autoradiography. Radioactive lipids can be located by the technique of autoradiography, exposing the chromatogram to a photographic emulsion in a conventional manner (27, 112) (Figs. 2, 3).

Enzymatic Test for Lecithin. The identification of lecithin (and phosphatidyl ethanolamine) may be confirmed by the use of snake venom phospholipase A, which preferentially hydrolyzes the β-linked fatty acid (145 to 151) to yield α-lysolecithins (or α-lysophosphatidylethanolamines). Paper chromatography before and after venom treatment will permit identification of the unknown phosphatides as the lyso-phosphatides produced are completely separated from each other and from the parent phosphatides. This technique is simple and fairly specific.

Choline plasmalogens are also acted upon by venom phospholipase A, but at a slower rate (151, 152). In this case, α-lysoplasmalogens are formed (145, 151). Gottfried and Rapport have recently employed this enzymatic method to prepare choline plasmalogens that are free from the diester analogue (152).

The specificity of action of venom phospholipase A has been a controversial and confusing subject, only recently elucidated to the satisfaction of most people in this field. It was early recognized that this enzyme released unsaturated fatty acids from natural lecithins (153, 154). Hanahan carried out a structural analysis

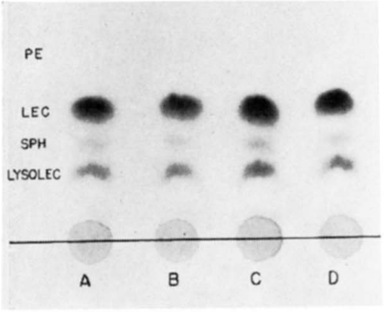

FIG. 2. Autoradiogram of P^{32}-labeled rat serum phosphatides. A rat was injected subcutaneously with 200 μc of P^{32}. After 24 hours, the animal was sacrificed and the serum obtained. Chromatography was carried out as explained in Figure 1. Autoradiography was done on X-ray film and required a 5-day exposure. A and B represent 20 μl of serum; C and D represent 30 μl of serum. The serum was applied directly to the silicic acid-impregnated paper. The spots at the origin represent serum proteins and water-soluble phosphate compounds.

of the lysolecithins formed and concluded on the basis of permanganate oxidation that they were β-lysolecithins and that the phospholipase had specifically removed the α-linked fatty acids (155). This work was apparently supported by Davidson *et al.* (156) and by Gray (157). However, Marinetti and co-workers (145) were unable to confirm the findings of these investigators and provided strong chemical evidence in the cases of both the choline and ethanolamine plasmalogens that the β-linked fatty acid was removed to yield α-lysoplasmalogens. It was recognized (151) that the problem of identification of the water-soluble phosphate esters of the hydrolyzed lysolecithins that had been treated with oxidants was difficult. During oxidation concomitant hydrolysis of lecithin and lysolecithin occurred and gave rise to unexpected water-soluble phosphate esters that were difficult to identify. These points have been discussed by Marinetti (151). By use of aqueous bromine oxidation of enzymatically formed lysolecithins, Marinetti *et al.* (148) were able to obtain the keto-lysolecithin derivative in yields of up to 92% at pH 7.4. At lower pH values the yield of keto-lysolecithin was diminished. The reason for this diminution at lower pH was not understood at first but is now believed to be caused by the cyclization of lysolecithin in acidic solution to form ortho-esters (158), protecting the secondary hydroxyl group from further oxidation. The high yield of keto-lysolecithin and the previous demonstration that this enzyme cleaved the β-linked fatty acid from plasmalogens gave evidence that venom phospholipase A acted to hydrolyze almost exclusively the β-linked fatty acid from these phosphatides. As stated in a previous paper (148), at no time was any evidence obtained for the presence of lysolecithinic acid.

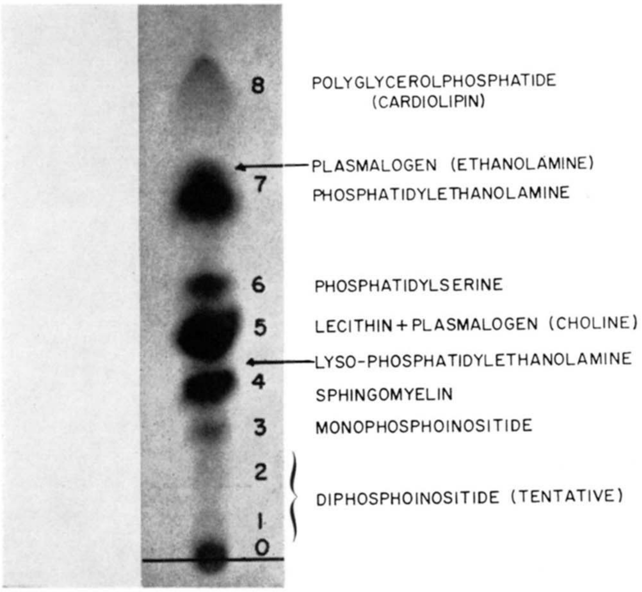

FIG. 3. Autoradiogram of P^{32}-labeled rat heart phosphatides. A rat was injected subcutaneously with 500 μc of P^{32}. After 48 hours, the animal was sacrificed and the heart ventricle lipids were extracted. Paper chromatography was carried out at 5° on a strip of silicic acid-impregnated paper 4 x 42 cm in a 1-liter graduate cylinder. The time of the run was 18 hours. The solvent system was diisobutyl ketone–acetic acid–water, 40:20:3 (v/v). Autoradiography was done on X-ray film and required a 2-week exposure. In this run, the plasmalogen form of PE moved ahead of the PE and can be seen as a cap on this spot. The PGP moved well ahead of PE and the PS was nicely separated from PE and LEC. The identification of the diphosphoinositides (DPI) is tentative. Lyso-LEC may also occur in spot 3.

The specificity of action of phospholipase A has since been clarified by the work of Tattrie (146), the reinvestigation of Hanahan *et al.* (149), and the work of de Haas and co-workers (147, 150). There now seems to be no doubt that, with the lecithins that have been studied, the venom enzyme hydrolyzes almost exclusively the β-linked fatty acid. One cannot rule out the possibility, however, that a small amount of cleavage also occurs at the α-ester linkage.[5]

The earlier conclusion of Marinetti *et al.* (159) regarding the chain length preference of phospholipase A must be considered to be in error on the basis of this newer work. The misinterpretation was based on an assumed specificity of action of this enzyme that was incorrect. It has now been shown by de Haas and van Deenen (150) that phospholipase A does not favor the hydrolysis of longer chain fatty acids or unsaturated acids; rather, the primary factor is the position of attachment of the fatty acid. Whether or not this specificity will hold true for other fatty acids on all the natural lecithins remains to be seen. Further details on the properties of phosphatidases can be found in the recent review article of Kates (160).

FACTORS AFFECTING THE CHROMATOGRAPHY OF PHOSPHATIDES ON SILICIC ACID-IMPREGNATED PAPER

Paper Impregnation. The papers must be uniformly impregnated in order to obtain reproducible chromatograms. In our experience, preparation of the sodium silicate solution as described here and elsewhere (113) has given more satisfactory results than may be obtained by the use of commercial sodium silicate solution.

Some of the papers impregnated as described here may have patchy irregularities or powdery areas. These papers can often be salvaged by gently rubbing off the excess silicic acid with clean tissue paper. At-

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[5] S. R. Bennett and N. H. Lattrie have recently (*Can. J. Biochem. Physiol.* **39**: 1357, 1961) published a paper that reconsiders this possibility.

tempts are under way to further improve impregnation by the addition of certain "binding" agents to the silicate solution. Silicic acid-impregnated paper may be available commercially in the near future (H. Reeve Angel and Co., Schleicher and Schuell Co.).

Humidity. The relative humidity of the environment has a marked influence on the mobility of the phosphatides. With increased humidity, the paper becomes more saturated with water, resulting in increased mobility of nearly all the lipids and, if pronounced, leading to overlapping and poor resolution. It is, therefore, advisable to store impregnated papers in a constant humidity environment. It is also desirable, but more difficult, to carry out the chromatography under conditions of controlled humidity.

Temperature. Reproducible chromatography is also dependent on constant environmental temperature, attained by running the chromatograms in a constant temperature room. We have found 23° a suitable temperature.

In some cases development should be performed at a lower temperature to minimize degradation of very labile compounds such as plasmalogens, or to effect a better resolution of some components. The solvent system must be modified for lower temperature development. At 0° to 5° the following solvents were found to be effective: (a) diisobutyl ketone-acetic acid-water, 40:20:3 (v/v); (b) diisobutyl ketone-*n*-butyl ether-acetic acid-water, 20:20:20:3 or 15:25:20:3 (v/v) (113). With some tissue lipids, low temperature chromatography permits partial and sometimes complete separation of the diester and plasmalogen forms of phosphatidyl ethanolamine (Fig. 3).

Application of the Lipids to the Paper. The lipids are applied to the paper in 10- to 30-μl aliquots. The amount applied per unit area is important; appropriate loads are given in the previous sections and are dependent on the size of the paper and the type of lipid mixture. Overloading must be avoided as it leads to spot elongation and trailing.

Once the lipids are applied to the paper, development should be carried out as soon as possible. Some lipids undergo changes if allowed to stay on the paper for long periods of time (a matter of hours) and may not chromatograph well.

Exposure of the Paper to Volatile Basic Solvents. The silicic acid-impregnated paper offers an active surface for adsorption of volatile compounds. Compounds that are adsorbed and that can influence the mobility of the lipids include ammonia, collidine, lutidine, pyridine, and other amines. In some cases, this may be desirable; in others, detrimental.

Storage. The effect of "aging" on the sodium silicate solution and on stored impregnated paper has not been adequately studied. In our experience, this factor probably has a very minor influence on the attainment of good chromatograms.

Hydrolysis of Lipids During Chromatography. Acidic or basic solvents might be expected to effect a hydrolysis of some phosphatides. In our experience, solvents containing acetic acid, ammonia, or pyridine cause very little if any such hydrolysis during the actual development of the chromatogram. Indeed, the ionization of acetic acid is so markedly diminished in organic solvents that its acidic properties become negligible in regard to lipid hydrolysis.

Binding of Applied Lipids at the Point of Application. Marinetti *et al.* (161, 162, 163) have noted that a small amount of P (1% to 3% of the total lipid P) remains at the origin. If commercial sodium silicate is used, as was the case when this work was begun (112), such binding at the origin can be appreciable. The use of sodium silicate made by treatment of analytical silicic acid with NaOH (113) minimizes this binding. Zieve *et al.* reported high values for binding of lecithin at the origin on silicic acid-impregnated papers and have suggested a way to circumvent it (164).

DIRECT PAPER CHROMATOGRAPHY OF LIPIDS WITHOUT SOLVENT EXTRACTION

It has been found (165) that lipids in some aqueous systems, such as plasma, serum, cytoplasm, or finely dispersed particles (mitochondria, microsomes, nuclei), can be chromatographed directly without prior extraction in organic solvents. Ten to 30 μl of plasma (or serum) is placed directly on silicic acid-impregnated paper, the aliquot is allowed to dry, and is then chromatographed in the usual manner. The phosphatides are separated in diisobutyl ketone-acetic acid-water (Figs. 1, 2). The neutral lipids (cholesterol esters, triglycerides, diglycerides, fatty acids, and free cholesterol) are separated in *n*-heptane-diisobutyl ketone, 96:6 (v/v); *n*-heptane-diisobutyl ketone-acetic acid, 96:6:0.5 (v/v); or *n*-heptane-benzene-acetic acid, 91:9:2 (v/v).

QUANTITATIVE ANALYSIS OF PHOSPHATIDES ON SILICIC ACID-IMPREGNATED FILTER PAPER

Quantitative analysis of the phosphatides may be carried out with paper chromatography (113, 123). In some cases the amount of lipid P in one spot is sufficient for analysis but it may be necessary to combine several spots in order to obtain enough P for accurate measurement. For amounts of P below 5 μg. the modified micromethod of Bartlett (32) is now used in place of the method of Chen *et al.* (33). Appropriate blank values are obtained from areas on the same chromatogram that correspond to the lipid spot in relative position and size (123).

Micro-Phosphorus Method (modified Bartlett procedure). The spots are eluted with methanolic-HCl as described previously (113, 123). After evaporation of the solvent, 0.9 ml of 70% perchloric acid is added. (*Nitric acid must not be used in this method.*) Digestion is carried out for 15 minutes on a medium gas flame or electric digestor. After the tubes have cooled, 7.0 ml of distilled water, 1.5 ml of 2.5% ammonium molybdate, and 0.2 ml of the aminonaphtholsulfonic acid reagent described by Bartlett (32) are added. The tubes are placed in boiling water for exactly 7 minutes, removed, and allowed to cool for 20 minutes. The optical densities are determined at 830 mμ. Appropriate blanks and standards must also be run. In our experience, this method is better than the vitamin-C procedure; the color is more stable, the blanks are lower, and the method is less susceptible to erratic high values. The optical density for 1 μg of P in a 1-cm cell path in the Beckman spectrophotometer is 0.105. The calibration curve is linear from 0.5 to 5 μg of P.

Semi-Micro Phosphorus Method. Several spots of each component are combined so that the total amount of P is between 10 and 40 μg. The spots are eluted with methanol-HCl as described above. In this case more extracting solvent will be needed (usually 10 to 20 ml for each extraction). The extracts are filtered into appropriate digestion flasks and evaporated.

Digestion is carried out with 0.5 ml of 70% perchloric acid and 2 drops of nitric acid for 15 minutes. After the flasks have cooled, 7.0 ml of distilled water is added. To each flask are then added 1.0 ml of 2.5% ammonium molybdate and 1 ml of elon reagent (34). The solutions are mixed and left at room temperature for 30 minutes. The optical density of each solution is determined at 820 mμ. Standard solutions are made to contain from 10 to 40 μg of P. Twenty micrograms of P gives an optical density reading of 0.3 in a 1-cm path.

QUANTITATIVE ANALYSIS OF PHOSPHATIDES ON SILICIC ACID-IMPREGNATED GLASS FIBER PAPER

Dieckert *et al.* (116, 117, 118), Brown *et al.* (120), and Muldrey *et al.* (121, 138) have developed chromatographic methods for phosphatide separation and analysis on silicic acid-impregnated glass fiber paper. Most of these studies have been done on model mixtures or on rather simple lipid mixtures. Lecithin, sphingomyelin, phosphatidyl ethanolamine, and lysolecithin are resolved and can be analyzed by a charring technique coupled with densitometry. It remains to be established how effective these systems are in separating more complex mixtures of phosphatides such as those obtained from the brain or heart. Charring, of course, is not specific.

In comparing the quantitative method of Muldrey *et al.* (121) with that of Marinetti *et al.* (113), the following points are noteworthy. (1) The charring technique appears to be simpler and more rapid. (2) The Marinetti technique gives the P content of each component and hence the amount of each phosphatide. This eliminates the standard calibration curves required for the charring technique. Indeed, it is not possible at the present to obtain precise calibration curves or precise standards since many of the phosphatides occurring in natural sources are not available in pure form. (3) The analysis for P is more precise than the measurement of the degree of charring by densitometry. (4) The Marinetti technique allows the determination of the specific radioactivity of the phosphatides per unit amount of P. (5) The measurement of lipid P gives supporting chemical evidence that the lipid in question is a phosphatide. (6) The charring technique is dependent upon a number of variables, such as temperature and time of heating, and shape and size of the spot. These variables are not critical when the absolute amount of P is determined. (7) Both methods depend on good resolution of components and on the homogeneity of each spot.

Chromatography on silicic acid-impregnated filter paper has other advantages over glass fiber paper. (1) The filter paper is more economical than glass fiber paper. (2) Filter paper is more manageable than glass fiber paper and, for example, can be rolled easily into cylinders for ascending chromatography. (3) The resolution of complex mixtures of phosphatides on silicic acid-impregnated paper has been superior to that obtained on glass fiber paper.

QUANTITATIVE ANALYSIS OF THE PHOSPHATIDES BY THE TRICOMPLEX STAINING METHOD

Hooghwinkel and co-workers (166) have used the chromatographic methods of Marinetti *et al.* (113) for quantitative paper chromatographic analysis of the phosphatides by the tricomplex staining procedure, in which an anionic dye is coupled to certain phosphatides by uranium cations. The complex is eluted from the paper and the optical density of the eluate is measured at 510 mμ. The procedure is based on a calculated combining ratio of dye with the phosphatide and its accuracy depends on quantitative and reproducible binding of the dye to the phosphatide as well as on the careful elution of the appropriate area of the paper. A limitation of this method is the failure of some phosphatides to complex with the dye.

QUANTITATIVE ANALYSIS OF PHOSPHATIDES ON CHROMATOGRAMS BY NEUTRON ACTIVATION

Benson and co-workers (167) and Strickland and Benson (168) have reported the analysis of phosphatides by neutron activation. The chromatogram of the partial hydrolysis products of the phosphatides (or of the intact phosphatides) is exposed to a high neutron flux which converts the P^{31} to P^{32}. After allowing other short-lived radioactive isotopes in the paper to decay, the radioactivity of the P^{32} is measured. By spotting known amounts of phosphate on the same papers, a quantitative estimation of the P in each spot can be made. The method is limited by the background radioactivity, by the uniformity of neutron flux and its ability to convert the same amount of P in each spot to radioactive P, and by the intensity of radioactivity that can be induced in each spot. This method appears to have considerable promise, but at the present time is not as precise or as sensitive as the Marinetti technique. We have sent chromatograms to Dr. Benson for neutron activation and have found that chromatograms that can be analyzed for P by our method do not yield sufficient radioactivity to be accurately analyzed by the method of Benson. If more radioactivity could be induced in these spots, the method of Benson would offer great promise as an analytical tool for phosphatides.

PROLONGED HYDROLYSIS OF THE PHOSPHATIDES

The phosphatides can be hydrolyzed completely to yield glycerol, serine, ethanolamine, choline, sphingosine, inositol, and fatty acids or aldehydes. These components can then be analyzed by methods already discussed or available in the literature (1 to 12). The limitations of this prolonged hydrolysis are apparent.

ANALYSIS OF PARTIAL HYDROLYSIS PRODUCTS OF PHOSPHATIDES

The previous sections have dealt with the analysis of the intact phosphatides. This section deals with the analysis of the phosphatides after partial alkaline hydrolysis or partial alkaline and acid hydrolysis. The chemical reactions involved in this procedure, which have been extensively studied by Dawson (64), are given in Figures 4 and 5 and in Tables 1 and 2. The phosphatides are hydrolyzed under specified mild conditions with methanolic-NaOH or with trichloracetic acid or acetic acid.[6] The resulting water-soluble phosphate esters are isolated and then separated by paper chromatography and ionophoresis. The quantitative analysis is carried out by elution of the spots and determination of the P in each. As some lipids resist alkaline hydrolysis or do not yield water-soluble products, Dawson has coupled mild acid hydrolysis with alkaline hydrolysis and has thus extended the usefulness of this technique.

Several points regarding this method are noteworthy. With respect to the mild alkaline hydrolysis of the diester glycerol phosphatides typified by structure I in Figure 4, the major water-soluble phosphate esters[7] formed are GPC, GPS, GPI, GPG, GPGPG, GP, and GPIP (Table 1). GPIP, which presumably should arise from diphosphoinositide, has not been well characterized and represents several isomers. Indeed, the structures of the diphosphoinositides and triphosphoinositides are still uncertain (12), and therefore the structures of the water-soluble phosphate esters that these phosphatides should yield are difficult to ascertain. Unless the conditions of the hydrolysis are carefully controlled, cyclic phosphate esters (VI, Fig. 4) form, complicating the identification and analysis of these compounds (65). Moreover, prolonged mild alkaline hydrolysis will cause the nitrogen base to be cleaved off yielding a mixture of α- and β-methyl glycerophosphates (VII and VIII, Fig. 4). Hence, from the standpoint of an analytical technique, this procedure is useful only if these secondary reactions are prevented. Recognizing this, Dawson found it necessary to correct the analysis for GPC because it gives rise to a measurable amount of cyclic GPC even when the reaction time is brief (64).

It is also apparent from Figures 4 and 5 that the lysophosphatide analogues (V, Fig. 4) of the respective parent diester or polyester phosphatides will yield the same partial hydrolysis product. Hence, no distinc-

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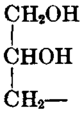
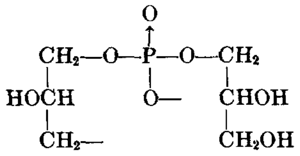
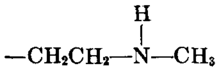
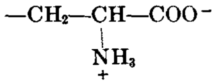
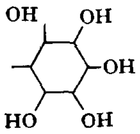
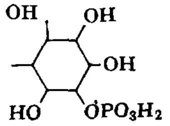
[6] In general, mild alkaline hydrolysis splits ester bonds and liberates fatty acids. In methanol, the fatty acids are cleaved off as methyl esters. Acetic acid hydrolysis cleaves the vinyl ether bond of plasmalogens to yield fatty aldehydes. This is not the only reaction that occurs, however, as phosphatides having a free hydroxyl group adjacent to the phosphate group may undergo cyclization and cleavage. Thus, polyglycerylphosphatide (cardiolipin) yields diglyceride and glyceryl diphosphate when treated with 90% acetic acid at 90° to 100°. Moreover, some hydroxy groups may be acetylated. A marked change has been observed in the mobility of inositol phosphatide when treated with acetic acid under these conditions.

[7] See footnote 1.

TABLE 1. WATER-SOLUBLE PHOSPHATE ESTERS OBTAINED BY MILD ALKALINE HYDROLYSIS OF THE PHOSPHATIDES*

Compound III†	Symbol for III†	Parent Phosphatide Giving Rise to III
R_3		
H—	GP	Phosphatidic acids, lysophosphatidic acids.
 $CH_2OH-CHOH-CH_2-$	GPG	Bis-phosphatidic acids‡ and lyso analogues.
	GPGPG	Polyglycerylphosphatide (cardiolipin) and lyso analogues.
$-CH_2CH_2-\underset{+}{N}-(CH_3)_3$	GPC	Lecithin, lysolecithin.
$-CH_2CH_2-N-(CH_3)_2$	DMGPE	N,N-Dimethylphosphatidylethanolamine, and lyso analogue.
 $-CH_2CH_2-\overset{H}{N}-CH_3$	MMGPE	N-Monomethylphosphatidylethanolamine, and lyso analogue.
$-CH_2CH_2\underset{+}{N}H_3$	GPE	Phosphatidylethanolamine, lysophosphatidylethanolamine.
 $-CH_2-\underset{\underset{+}{NH_3}}{CH}-COO^-$	GPS	Phosphatidylserine, lysophosphatidylserine.
	GPI	Monophosphoinositide and lyso analogue.
	GPIP§	Diphosphoinositide and lyso analogue.
Inositol diphosphate	$GPIP_2$	Triphosphoinositide and lyso analogue.

* Hydrolysis with NaOH in methanol at 37° for 15 minutes as described by Dawson (64).

† See Figures 4 and 5 for the meaning of R_3. All compounds represented by III will be considered to be L-isomers. See footnote 1 for meaning of symbols.

‡ Also named phosphatidylglycerol.

§ Tentative structure. Various isomers may occur.

tion can be made between these phosphatides, as pointed out by Dawson (64). If the lysophosphatides occur in small amounts, it may be relatively safe to ignore them. In serum and possibly in other tissues, such as abnormal red blood cells, the lysophosphatides may occur in appreciable amounts and cannot be ignored (123, 169, 170) (see also Figs. 1 and 2).

The plasmalogens (typified by structures II, X, and XI, Figs. 4 and 5) may or may not be converted to water-soluble phosphate esters depending on the conditions of hydrolysis. The ether-type phosphatides shown by structures IX, XI, and XII (Fig. 5) do not yield water-soluble phosphate esters either under mild alkaline or acid hydrolysis. Phosphatides of the type IX and XI have not yet been characterized. It is apparent that plasmalogens depicted by II (Fig. 4) yield water-soluble phosphate esters only if *both* acid and alkaline hydrolysis are employed. The saturated diether phosphatides (XII, Fig. 5) are stable to both acid and alkaline hydrolysis and hence behave like sphingomyelin (XIV). Indeed, the glycerol ether analogue (XIII) of lyso-PE was mistaken for a new type of sphingomyelin by Carter *et al.* (171) when this lipid was first isolated. A similar lipid has more recently been obtained by Svennerholm (172). Phosphatides of this type have also been synthesized by Bevan and Malkin (173). Phosphatides of the type IX yield lyso-phosphatides of the ether-type XIII when treated with alkali, whereas phosphatides of the type XI yield similar lysophosphatides (XIII) when treated with mild acid.

The divinylether plasmalogens (X, Fig. 5) are converted to water-soluble phosphate esters (III, Fig. 4) by mild acid hydrolysis but are stable to alkaline hydrolysis. Their recognition, therefore, depends on the sequence of hydrolytic steps employed. If only alkaline hydrolysis is used, they may be taken for sphingomyelin or at least be included with the sphingomyelin fraction. If only acid hydrolysis is employed, they will be converted to water-soluble esters. If both acid and alkaline hydrolyses are performed in sequence, they will be measured with, and hence be indistinguishable from, the typical monovinylether plasmalogens (II). Divinylether plasmalogens (or the saturated diether phosphatides) of the choline and ethanolamine type are believed to occur in small amounts in beef and pig heart (145). The evidence for their occurrence is based on the isolation of glycerol diethers (174). These glycerol diethers were originally mistaken for β-glycerol monoethers. Evidence for the occurrence of plasmalogen analogues of cardiolipin, lecithin, PE, PS, MPI, and possibly DPI has been reported by Hack (141).

In comparing the Dawson technique with methods that depend on the analysis of the intact phosphatides, several points are noteworthy. The properties conferred upon the intact phosphatides by the fatty acids, aldehydes, or ethers can be used to advantage to effect their separation. As the nature of these side chains must ultimately be determined before the complete

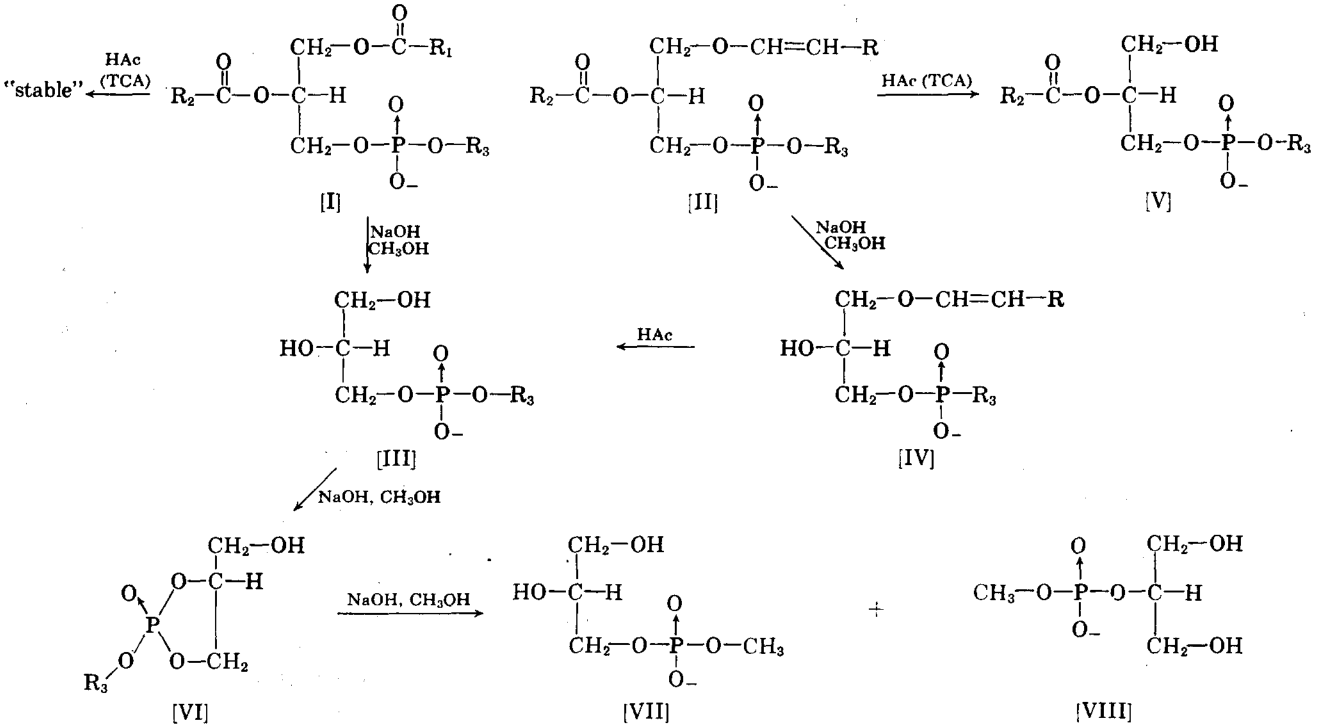


FIG. 4. Reaction scheme showing the mild alkaline and mild acid hydrolysis of the more abundant glycerylphosphatides. Mild alkaline hydrolysis is carried out with methanolic NaOH at 37° for 15 minutes as described by Dawson (64). R, R_1, and R_2 represent hydrocarbon chains that vary with respect to chain length, unsaturation, and number of functional groups. R_3 designates the N-bases (such as choline, serine, and ethanolamine), the alcohols (such as glycerol and inositol), or larger moieties (such as diglycerides, bis-phosphatidic acids, or peptides). These groups are shown in Table 1. The use of the term "stable" here designates the failure to obtain water-soluble phosphate esters under specified conditions and does not necessarily mean that no reaction occurs. Thus, the vinyl ether plasmalogens may undergo cyclization in alkaline solution. The hydrolysis with acetic acid is carried out as reported by Dawson (64). This treatment cleaves off the aldehyde group of the vinyl ether plasmalogens (II), and also may partially acetylate the free hydroxyl groups of some phosphatides. Acetic acid hydrolysis also hydrolyzes the cardiolipins to yield GPG and diglycerides as major products (207). The compounds represented by (III) are L-isomers.

structure of a phosphatide can be obtained, the isolation of intact phosphatides must eventually be achieved. The side chains can also be used to develop detection methods for the phosphatides (such as dye binding, reaction with iodine and permanganate, reaction with the Schiff reagent, enzymatic cleavage, etc.).

The Dawson hydrolysis technique must be carried out under carefully controlled conditions to prevent secondary reactions that would make the method useless.

It has the advantage that certain water-soluble phosphate esters are easier to separate than the corresponding parent phosphatides from which they are derived. Thus, GPE and GPS are more easily separated than the parent phosphatides PE and PS. Furthermore, GPG and GPGPG are more easily resolved than phosphatidyl glycerol (bis-phosphatidic acid) and polyglycerolphosphatide (cardiolipin). The analysis of P in the spots of the water-soluble phosphate esters is more easily carried out than the analysis of P in the phosphatide spots because the latter must be eluted from the silicic acid-impregnated paper before the analysis is done. The detection of the spots and the actual cutting out of the areas corresponding to the components in question are more precise in the case of the intact phosphatides, however, because the phosphatides can be localized beforehand by staining with Rhodamine 6G.

The data summarized in Tables 1 and 2 condense the major features of the Dawson partial hydrolytic methods and the paper chromatographic procedure of Marinetti *et al.* The combined use of these two paper chromatographic methods yields much valuable data and is extremely useful for the separation, identification, and analysis of phosphatides.

It should be clear from the above that the isolation and identification of all phosphatides that might possibly occur in a given tissue can be a complex and difficult problem. There are at least six possible classes of

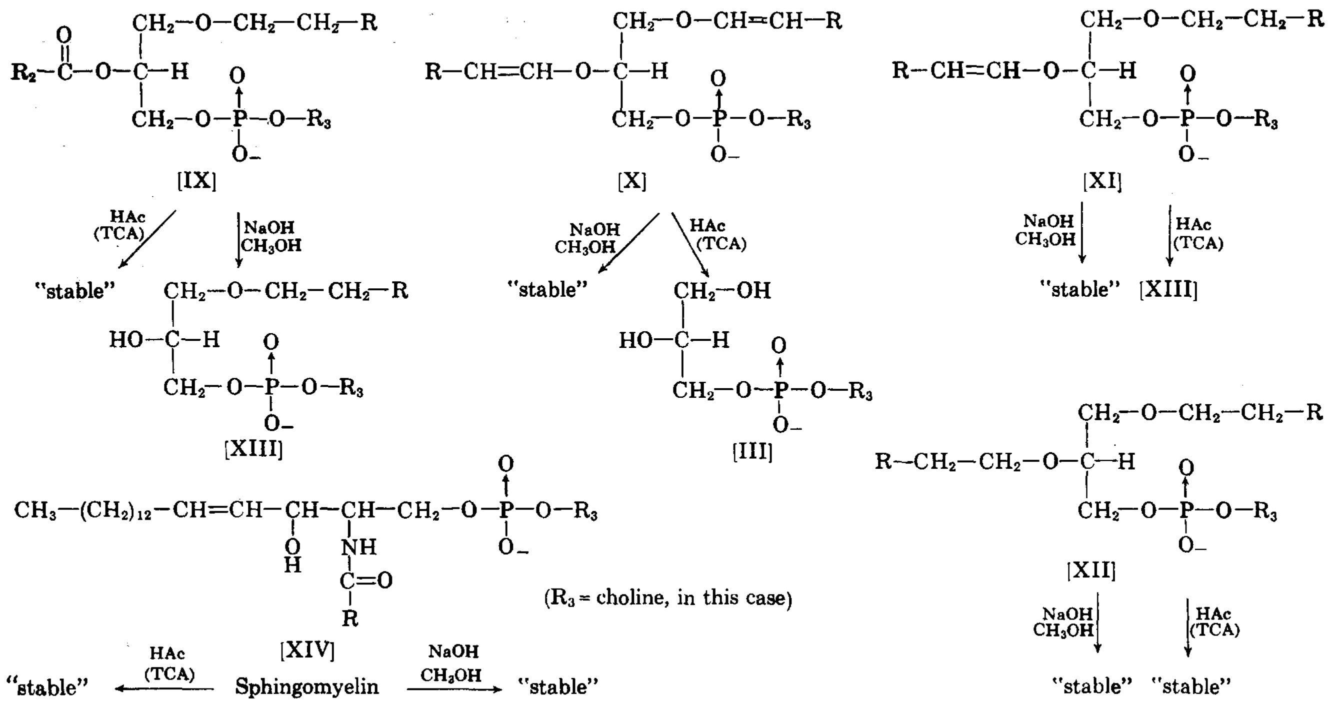


Fig. 5. Reaction scheme showing the mild alkaline and mild acid hydrolysis of the less abundant glycerylphosphatides and of sphingomyelin. See Figure 4 for details of the hydrolysis. Evidence for all of these phosphatides has not yet been reported in the literature. Phosphatides of type XIII (and by inference IX) have been isolated by Carter *et al.* (171) and Svennerholm (172). Phosphatides of the type X, XI, and XII are proposed on the basis of the work of Marinetti *et al.* (174).

glycerol phosphatides based on their fatty acid, fatty aldehyde, or fatty ether composition. Each of these six classes will have corresponding lyso analogues. Furthermore, each class represents a family of compounds since the fatty acid, aldehyde, or ether may vary with respect to length of side chain, degree of unsaturation, and functional groups. Each class can be further subdivided into at least nine subgroups based on the R_3 group that is esterified to the phosphate moiety of glycerol (i.e., R_3 may be choline, serine, ethanolamine, etc.) (Table 1). The total number of possible phosphatides is quite large. Although the methods presently available represent remarkable achievements in lipid technology, there is a long road ahead before methods can be developed to permit the separation, identification, and analysis of all possible phosphatides. The situation may be even more complex in some plants and bacteria.

COLUMN CHROMATOGRAPHIC SEPARATION OF PHOSPHATIDES AND PARTIAL HYDROLYSIS PRODUCTS

The use of column chromatography for the separation of phosphatides is included primarily for the sake of completeness and is discussed very briefly. Initially, columns of magnesium oxide (175), magnesol-celite (176), alumina (177, 178, 179), and cellulose (94, 180) were used. More recent work has utilized columns of silicic acid (24, 113, 181, 182, 183), florisil (184), and diethylaminoethyl (DEAE) cellulose (114). In general, silicic acid columns have found the widest application. The phosphatides are separated into groups rather than into individual components. It is not usually possible to obtain a pure phosphatide by this method when one starts with a total lipid extract from a tissue unless the phosphatide composition is quite simple as, for example, in egg yolk. Thus, it is usually necessary to fractionate the total lipid mixture by some other means before column chromatography or to use several column fractionation procedures. The obvious advantage of column chromatography is that it allows for the preparation of sufficient material for extensive chemical analysis. The purification of PE and PS (114, 185 to 188) by column chromatography has been reported. Furthermore, the free acid and salt form of PS have been resolved on silicic acid (187).

We have found it extremely informative and nearly obligatory to examine all column fractions by paper chromatography in order to ascertain their purity. The column fraction may be eluted as a symmetrical peak and give acceptable elementary analysis but yet not be pure. Supposedly pure fractions of phospha-

TABLE 2. PROPERTIES OF THE PHOSPHATIDES[*]

		Reaction in Spot Tests[‡]				Products Obtained on Mild Hydrolysis[§]	
Phosphatide	R_f[†]	Rh.6G	Ninh.	DNPH	Choline	HAc or TCA	NaOH-CH_3OH
1. Lecithin	0.37	yel	−	−	+	−	GPC
2. Lysolecithin	0.18	yel	−	−	+	−	GPC
3. PE	0.50	yel	+	−	−	−	GPE
4. Lyso-PE	0.30	yel	+	−	−	−	GPE
5. Monomethyl-PE	0.50	yel	−	−	−	−	MMGPE
6. Dimethyl-PE	0.48	yel	−	−	−	−	DMGPE
7. PS	0.45	blue	+	−	−	−	GPS
8. Lyso-PS	0.25	blue	+	−	−	−	GPS
9. MPI	0.21	blue	−	−	−	−	GPI
10. DPI[‖]	(0.16)	blue	−	−	−	−	GPIP
11. Choline-Plas.	0.38	yel	−	+	+	lysolec.	lysoplasmal.
12. Et-Plas.	0.51	yel	+	+	−	lyso-PE	lysoplasmal.
13. Serine-Plas.[‖]	(0.46)	(?)	+	+	−	lyso-PS	lysoplasmal.
14. Lysoplas.-Ch.	0.21	yel	−	+	+	GPC	−
15. Lysoplas.-Et.	0.32	yel	+	+	−	GPE	−
16. Lysoplas.-Ser.[‖]	(0.27)	(?)	+	+	−	GPS	−
17. Phosphat. Acid	0.78	blue	−	−	−	(?)	GP
18. Bis-phos. Acid	0.57–0.67	blue	−	−	−	(?)	GPG
19. Cardiolipin[#]		blue	−	−	−	GPG + Digl.	GPGPG
20. Sphingomyelin	0.31	yel	−	−	+	−	−
21. Divinylplas-Ch.[‖]	(0.38)	(yel)	−	+	+	GPC	−
22. Divinylplas-Et.[‖]	(0.51)	(yel)	+	+	−	GPE	−
23. Dietherplas-Ch.[‖]	(0.38)	(yel)	−	−	+	−	−
24. Dietherplas-Et.[‖]	(0.51)	(yel)	+	−	−	−	−

[*] See Table 1 and text footnote 1 for the meaning of the symbols.

[†] R_f values given here are approximate values as obtained by chromatography on silicic acid-impregnated paper in 2-qt. Mason jars as described here and elsewhere (206). The solvent was diisobutyl ketone-acetic acid-water, 40:20:3 (v/v). The temperature was 23°. Pure synthetic phosphatides corresponding to 1, 3, 5, 6, 17, and 18 were tested. These samples were obtained from Dr. E. Baer, University of Toronto. Highly purified samples of phosphatides corresponding to 2, 4, 7, 8, 9, 11, 12, 14, 15, 19, and 20 were prepared and tested in this laboratory.

[‡] Staining with Rhodamine 6G = Rh.6G; staining with ninhydrin = Ninh.; staining with dinitrophenylhydrazine = DNPH; reaction for choline = Choline.

[§] Mild hydrolysis by the Dawson technique (64) as explained in this paper.

[‖] These compounds have not been chromatographed. Their R_f values and staining with Rhodamine 6G are estimates based on the behavior of corresponding analogous phosphatides.

[#] Also called polyglycerylphosphatide.

tides, obtained from reputable biochemical supply companies and lipid chemists, have usually been found to be heterogeneous. For example, we examined pure synthetic lecithin from two biochemical companies and in both cases found them to contain at least four components, a major one being lysolecithin. Two synthetic samples of phosphatidyl ethanolamine were also examined by paper chromatography and in each case lyso-PE was present. One synthetic PE sample was almost chromatographically pure, containing only a very small amount of lyso-PE. We also found synthetic phosphatidic acids to be heterogeneous. In some cases the phosphatidic acid was obtained as the free acid, and extensive autohydrolysis had produced a considerable amount of lysophosphatidic acid and free fatty acids. The fault here was not due to the supplier but to the instability of these acidic phosphatides. We have also examined by paper chromatography several preparations of inositol phosphatide made by the Folch procedure or by column chromatography, and in every case they were heterogeneous.

The separation of the water-soluble phosphate esters obtained by partial hydrolysis of phosphatides has also been achieved by column chromatography on ion exchange resins (189). This development extends the paper chromatographic method of Dawson (64), allows for the preparation and further characterization of some of the minor and as yet unidentified components, and may lead to the elucidation of the structure of new phosphatides.

RECENT STUDIES

Paper Chromatography. The author has recently examined modified filter paper (cellulose phosphate, carboxycellulose, DEAE cellulose) and filter paper impregnated with anionic and cationic resins or with $ZnCO_3$, $CaCO_3$, and $BaSO_4$ (unpublished data). These papers have less resolving power than silicic acid-impregnated paper but do give useful separations with certain simple mixtures. In particular, the aminophosphatides are well separated. The R_f values for PE, PS, lyso-PE, lyso-PS, and lecithin are given in Table 3. The solvent system used was the same as that employed for silicic acid-impregnated filter paper, namely diisobutyl ketone-acetic acid-water, 40:20:3 (v/v). As shown in Table 3, a resolution of PE, PS, lyso-PE, and lyso-PS can be achieved. However, PE cannot be separated from lecithin, and lecithin cannot be separated from sphingomyelin. These systems can be used in conjunction with silicic acid-impregnated papers for the identification of the aminophosphatides.

Separations were also obtained on papers impregnated with anionic resins and with insoluble carbonate or sulfate salts, but these were not as satisfactory as those obtained on the papers given in Table 3.

Mild Alkaline and Acid Hydrolysis. The deacylation of phosphatides (and neutral lipids) has now been achieved in our laboratory (unpublished data) by the use of methanolic sodium methoxide at room temperature or at 0°. A mixture of lipids in chloroform-methanol 1:1 is treated with 0.5 N sodium methoxide in methanol (0.2 ml of 0.5 N sodium methoxide in methanol added to 0.8 ml of lipid solution in chloroform-methanol containing 5 mg of lipid). The ester phosphatides are completely deacylated at room temperature within 10 minutes to yield fatty acid methyl esters and the corresponding water-soluble phosphate esters. These reactions are shown in Figure 6. Indeed, by carrying out the reaction at 0°, we have been able to isolate and prepare the lyso derivatives of lecithin, PE, and PS. This reaction has also been used to prepare mixed di- and monoglycerides from triglycerides. A time study of hydrolysis of purified egg lecithin at 0° is shown in Figure 7. The maximum yield of lysolecithin occurs between 4 and 5 minutes and is about 35% to 40% of the original lecithin. We have investigated the fatty acids released and have shown that either the alpha or beta acids are removed to yield a mixture of α- and β-lysolecithins. There is a small preferential formation of β-lysolecithins. Sphingomyelin and cerebrosides are stable toward this reagent, and the plasmalogens of the monovinylether type (II, Fig. 4) are converted to their corresponding lysoplasmalogens (IV).

TABLE 3. R_F VALUES FOR PHOSPHATIDES CHROMATOGRAPHED ON DIFFERENT KINDS OF PAPER*

Phosphatide	Carboxymethyl Cellulose†	Cellulose Phosphate†	Amberlite IRC-50†
Lecithin	0.83	0.83	0.72
PE	0.78	0.79	0.71
PS	0.54	0.66	0.56
Lyso-PE	0.40	0.57	0.50
Lyso-PS	0.26	0.45	0.41

* Paper chromatography carried out in 2-qt. Mason jars. The chromatographic solvent was diisobutyl ketone-acetic acid-water, 40:20:3 (v/v). Temperature of the run was 23°. Time of the run was 3 hours. Ten micrograms of each lipid was used.

† These impregnated cellulose papers were obtained from H. Reeve Angel and Co.

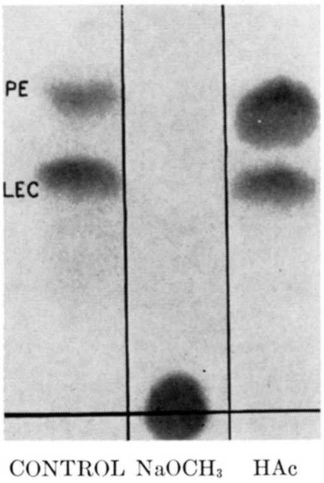


FIG. 6. Autoradiogram of P^{32}-labeled rat liver phosphatides. Rat liver phosphatides were labeled *in vivo* by injecting a rat subcutaneously with 200 μc of P^{32}. The animal was sacrificed after 12 hours and the liver lipids were extracted. A sample of the liver lipids was divided into three equal parts: (1) the control, (2) one treated with sodium methoxide in chloroform-methanol, 1:1 (v/v) at 23° for 20 minutes, and (3) one treated with 90% acetic acid-mercuric chloride solution at 95° for 30 minutes. Aliquots of each solution were spotted on silicic acid-impregnated paper and chromatography carried out as explained in Figure 1. About 1 μg of lipid P was placed on each spot. These chromatograms were not stained with Rhodamine 6G so as to avoid the loss of the water-soluble phosphate esters formed as a result of the sodium methoxide treatment. Autoradiography was done on X-ray film and required a 6-day exposure. All the ester phosphatides were altered by the methoxide hydrolysis. The autoradiogram shown here depicts only the major phosphatides, namely PE (upper spot on the left and right chromatograms) and lecithin (the spot just below PE). The other phosphatide components such as PGP, PS, MPI, and SPH did not have sufficient radioactivity to be clearly seen on the photograph shown here. These components were easily detected by staining with Rhodamine 6G. The origin spot in the center chromatogram represents the water-soluble phosphate esters (GPC, GPE, GPS) formed by methoxide hydrolysis. In this run, only sphingomyelin (which was barely visible) resisted hydrolysis and hence its mobility was unaltered. The acetic acid hydrolysis shown by the right run did not affect the mobility of PE and lecithin. Although not visible on these autoradiograms, both PGP and MPI were affected. PGP was hydrolyzed in major part to a diglyceride and water-soluble phosphate ester. The change in MPI has not yet been elucidated.

The water-soluble phosphate esters (III) formed by the methoxide hydrolysis can be placed directly on filter paper and separated by the methods described by Dawson (64) and by Maruo and Benson (65). There is no need to neutralize the methoxide or to isolate the water-soluble phosphate esters before chromatographing them. This method, therefore, is simpler to carry out. It is important to keep the reaction time below 30 minutes, otherwise the N-base of the phosphate esters is split off. We were able to identify GPC, GPS, GPE, and GPI as hydrolytic products. GPG, GPGPG, and GP occurred in very small amounts and were not detected on the chromatograms by autoradiography. It is noteworthy that these phosphate esters remain at the origin on the silicic acid-impregnated papers (Fig. 6).

When the phosphatides are hydrolyzed with acetic acid containing mercuric chloride (reaction time 30 minutes at 90°; 5 mg of phosphatide dissolved in 1.0 ml of 90% acetic acid and 1 drop of saturated $HgCl_2$), the plasmalogens are hydrolyzed to lysophosphatides. This reaction coupled with paper chromatography may be used for the analysis of plasmalogens. The polyglycerylphosphatides, however, are hydrolyzed by this treatment to yield diglycerides and other products, and the inositol phosphatides are altered so that they move with PE on paper chromatograms.

Chemical Production of Mixed Lysophosphatides. The controlled sodium methoxide hydrolysis of lecithin, PE, and PS permits the synthesis of lysolecithin, lyso-PE, and lyso-PS so that they can be used as chromatographic standards. At the present time these lysophosphatides may also be obtained in small yield as byproducts in the chemical synthesis of phosphatides; in the case of lecithin and PE, they can be obtained by enzymatic hydrolysis with venom phospholipase A. Venom hydrolysis yields primarily α-lysolecithin and α-lyso-PE, whereas chemical hydrolysis with methoxide yields a mixture of α- and β-lysophosphatides.

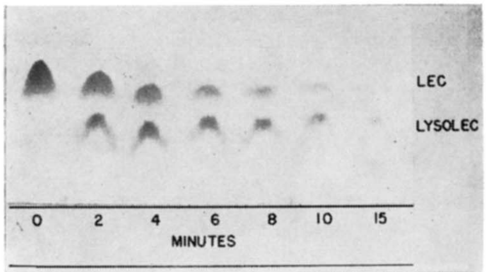


FIG. 7. Time course of reaction of purified egg lecithin with sodium methoxide at 0°. Eight milligrams of purified egg lecithin was dissolved in 0.8 ml of chloroform-methanol, 1:1 (v/v) and placed on ice. Then 0.5 ml of ice-cold 0.5 N sodium methoxide in methanol was added. At the times indicated in the figure, 10 μl was quickly removed and spotted on silicic acid-impregnated paper. Chromatography was carried out in Mason jars as described in Figure 1. The lipid spots were detected with the choline test (phosphomolybdic acid-$SnCl_2$) described in the text. The course of the reaction was easily followed by this procedure.

DISCUSSION

This review has dealt in some detail with two major methods for the separation, isolation, identification, and analysis of phosphatides. Both methods fall short of the ultimate aim of any analytical procedure. They are, however, the best micromethods available today and are yielding much useful information. When coupled with column chromatography and other techniques available for identification of organic compounds (e.g., infrared analysis [190, 191, 192], gas-liquid chromatography [85 to 90], nuclear magnetic resonance [171, 193], mass spectrometry [194], spectrophotometry, and chemical analysis), these paper chromatographic methods become extremely informative.

The identification of a specific phosphatide will in most cases require several techniques. For example, a phosphatide can be isolated by column chromatography or by solvent crystallization. The purity and identity of the isolated product can then be achieved in the following way: (1) elementary analysis; (2) determination of ester and aldehyde content and calculation of the ester : P and aldehyde : P ratios; (3) partial hydrolysis with acid or base, and characterization of the deacylated, water-soluble phosphate ester and the liberated fatty acids and aldehydes (or ethers); (4) complete hydrolysis and analysis for glycerol, serine, ethanolamine, choline, etc.; (5) paper chromatographic analysis (and spot tests) of the intact phosphatide in several solvent systems both before and after mild acid and alkaline hydrolysis and after enzymatic hydrolysis; and (6) analysis of the phosphatide by infrared and ultraviolet spectroscopy and, if necessary, by nuclear magnetic resonance or mass spectrometry.

In general, it is neither necessary nor possible to carry out all the above analyses. A combination of paper chromatography of the intact phosphatide and analysis by the Dawson technique, however, will yield much information in a rapid, simple, and effective manner. In most cases, these two techniques, either alone or in combination, can identify the major phosphatides of a given tissue. It is understood that this approach may not be unequivocal and that it will only identify the type of phosphatide (such as lecithin, phosphatidyl ethanolamine, etc.). It will not reveal the number of molecular species present in the sample. This can only be approximated by a careful and detailed analysis of the fatty acids, aldehydes, and ethers present.

Demands for identification are more rigorous when exploring a new biological source because the phosphatide may have an entirely different structure and may contain as yet unidentified components. The existence of such phosphatides has been reported by several laboratories. N-monomethyl and N,N-dimethyl phosphatidyl ethanolamine have been described by Artom (195) and by Bremer and Greenberg (196); and the occurrence of phosphatides containing glutamic acid (197), threonine (198), and other amino acids or peptides (199 to 205) seems very probable. Once a phosphatide has been isolated from a given source and the method of preparation is shown to be reliable, extensive identification of this same phosphatide every time it is made is unnecessary. Thus, the preparation of lecithin from egg yolk is a fairly simple procedure and an adequate identification can easily be made by N and P analyses, paper chromatography, and spot tests.

Although Figures 4 and 5 and Tables 1 and 2 indicate the occurrence of a large number of phosphatides, it should be emphasized that only a few of these occur as major components in most animal tissues. The major phosphatides are lecithin, sphingomyelin, phosphatidyl ethanolamine, monophosphoinositide, phosphatidylserine, and polyglycerylphosphatide (cardiolipin). The amount of each will vary from one tissue to another. There may be exceptions to these generalities, as evidenced by the finding that the red cells of ruminants lack lecithin (208). Cardiolipin has been found to occur in highest amount in heart muscle but is barely detectable in red blood cells and plasma. Cardiolipin is localized in the mitochondrial fraction of cells (161, 168).

The lysophosphatides are usually very minor constituents in tissues (27) but do occur in certain natural products. Thus, lysolecithin occurs in appreciable concentration in plasma (123, 209, 210) and lysophosphatidylethanolamine may also be present in plasma (123).

Plasmalogens of the monovinyl ether, monoester type (structure II, Fig. 4) are found in high concentration in heart, brain, and testes. They are, however, minor components in most of the other tissues. Plasmalogens of the type shown by structures X and XI (Fig. 5) and the ether-type phosphatides designated by structures IX and XII are trace components. These latter phosphatides have not yet been isolated from tissues, although the lyso-monoether phosphatide analogue (XIII) of structure IX has been characterized by Carter *et al.* (171). The isolation of a diether glycerol by Marinetti and co-workers (145) demonstrates the presence of phosphatides of the type X, XI, or XII in beef and pig heart.

Phosphatidic acids occur in trace amount in cells (64, 211, 212). The major problem here is whether they exist normally in the concentration range measured or whether they arise as breakdown products of other phosphatides. Dawson (64) has pointed out that GPC and other phosphate esters can give rise to GP under conditions of mild alkaline hydrolysis; if this method is used to measure phosphatidic acids, some correction must be made in the analysis of the data.

The minor phosphatides that occur in tissues are receiving more attention as it is recognized that some of these may have an important metabolic function. The role of phosphatidic acids in lipid metabolism (213) and their postulated role in membrane transport (214) has already been suggested.

The paper chromatographic methods discussed in this review have given evidence for the existence of as yet unidentified phosphatides. They have been important tools for the qualitative and quantitative analysis of phosphatides and have provided techniques for the discovery of new phosphatides. These methods should prove valuable in the study of abnormal metabolic states involving these lipids.

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