Glass Paper Chromatography of Lipids[1]

JAMES G. HAMILTON and JAMES E. MULDREY, Department of Biochemistry and the Nutrition and Metabolism Research Laboratory of the Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana

MODERN ADSORPTION CHROMATOGRAPHY of lipids began with Trappe's studies (1938 to 1941) of adsorbents and the "eluotropic series" (1) and with his early adsorption column methods for serum lipids (2, 3, 4). The first instance of quantitative elution of serum phospholipids was provided by Borgström's silicic acid column separations in 1952 (5) of neutral lipids and fatty acids from phospholipids. In 1952 Borgström also described the silicic acid column separation of cholesteryl palmitate from triglycerides and fatty acids (6). The elegant stepwise elution of serum lipids from silicic acid columns by Fillerup and Mead in 1953 (7) separated the major lipid classes: cholesteryl esters, triglycerides, cholesterol, fatty acids, and phospholipids. Then, with Borgström's report in 1954 (8) of the silicic acid column separation of tri-, di-, and monoglycerides, all of the information was at hand for for the column chromatography of the principal classes of neutral lipids of serum.

The advantages of adsorption chromatography were superimposed upon the conveniences of paper chromatography by Kirchner and Keller (9) in 1950, when they impregnated cellulose papers with silicic acid and used these papers for separating the dinitrophenylhydrazones of a series of methyl ketones. The chromatostrip, a thin layer of silicic acid on a glass plate, which enjoyed the added features of resistance to corrosive reagents and to high temperature, was introduced by Kirchner, Miller and Keller (10) in 1951 for chromatography of essential oils. The chromatostrip foreshadowed two important chromatographic media, the thin layer developed by Stahl (11) and the silicic acid impregnated glass fiber papers of Dieckert and Reiser (12). Strain (13) employed glass paper for separating carotenoids in 1953. A silicic acid impregnated cellulose paper was used by Lea and Rhodes (14) in 1954 for the separation of phosphatides. Simultaneously Dieckert and Reiser used silicic acid impregnated glass paper for the separation of neutral lipids (12, 15)). The glass paper technique was developed as a rapid and sensitive assay for the lipids in fractions eluted from silicic acid columns. In 1956 phosphatides were separated on silicic acid impregnated glass paper by Dieckert and Reiser (16) and on silicic acid impregnated cellulose paper by Witter, Marinetti, and Stotz (17).

Glass paper chromatography of the various lipids begins with the selection of the glass paper and the type of impregnation. After the glass papers have been given the appropriate treatment, suitable volumes of the respective lipid solutions are applied to marked spots. After the spotting solvent has evaporated the papers are suspended with the lower edge in the appropriate developing solvent. The chromatography is usually complete in approximately 5 to 20 min. After the developing solvent is removed the lipids are located by counting, by radioautography, by selective staining with group-specific reagents such as ninhydrin, or by the very sensitive and non-specific technique of charring the lipids with sulfuric acid and heat. Quantitative assay of the lipids may be accomplished by photometry of the charred residues on the glass papers.

Impregnated Glass Fiber Papers. There are a number of glass fiber papers available for possible use for chromatography. The present authors' experience has been limited to a paper manufactured by the Hurlbut Paper Co. (934-AH), Whatman glass papers (GFA, GFB, and GFC) all marketed by H. Reeve Angel, and to a paper manufactured by the Gelman Instrument Co., Chelsea, Mich. Although all of the papers, after impregnation, could be used for chromatographic separations, our preference is the Hurlbut paper. The Whatman papers required a longer time for developing in a solvent and were more expensive than 934-AH. The Gelman paper was too coarse and nonuniform to be generally useful. Recently an experimental glass paper, Hurlbut 934-RA, which is more uniform than 934-AH, has been made available to us for evaluation by Mr. Marshall Peterson of H. Reeve Angel. The desirability of a photometrically uniform glass paper will be pointed out in the section on quantitative technique.

Two different impregnations of glass paper with silicic acid have been reported by Dieckert and Reiser (15) and Dieckert, Carney, Ory and Morris (18). Potassium silicate paper was first prepared by Dieckert, Morris, and Mason (19) from silicic acid and potassium hydroxide. A similar paper was prepared by Muldrey *et al.* (20) from Mallinckrodt's sodium silicate. A potassium silicate, approximately 30% W/V, which is superior in uniformity and stability, is available from the Electrochemicals Dept., E. I. DuPont Co., Wilmington, Del. (Potassium Silicate, Electronics 200).[2]

The glass paper is dipped in potassium silicate of the proper concentration, the excess is drained off by passing a clean glass rod across both surfaces of the paper, and the paper is suspended over a hot plate until dry. Monopotassium phosphate paper (21), alumina paper (22), and silica gel paper (23) are currently prepared as previously described. Prepared papers are stored in aluminum foil until needed.

For the application of lipid extracts the impregnated paper is laid on a clean glass plate and is marked lightly with pencil about 2 cm from the bottom edge of the paper. Ten μl of lipid extract is applied to each marked spot, giving amounts of the desired lipid rangiing from 0.2 to 2.0 μg per spot. Serum may be applied to the glass paper without prior preparation of an extract. In this case, 1 μl to 10 μl of serum is pipetted onto a marked spot and is overspotted with 10 μl of chloroform-methanol (2:1

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[2] The availability of this silicate was kindly pointed out to us by Dr. J. W. Dieckert.

v/v) before drying. A second application of 10 μl of chloroform-methanol is made over the spot after the first has evaporated. Although lipid extracts can be applied successfully with a minimum of experience, the application and overspotting of serum requires practice to obtain symmetrical spots. It would seem that the direct spotting of serum should be limited to chromatography of neutral lipids and that conventional lipid extracts, freed of glucose, amino acids, urea, etc., should be used for phosphatide chromatography, since many of these nonlipid materials interfere with the separation of phosphatides.

After the spotting solvent has dried the paper is developed by ascending chromatography in the appropriate solvent. The chromatography tank is covered to minimize solvent evaporation, but no equilibration of the paper with the solvent vapor is necessary. For example, papers 9 x 15 cm. are developed in 1,000 ml. beakers by introducing solvent to a depth of a few mm., standing the glass paper in the beaker, and covering with aluminum foil. The developing time for this arrangement is approximately 11 min.

After development the chromatogram is removed, dried, and sprayed with reagent grade concentrated sulfuric acid so that both sides are evenly but not heavily coated. The sprayed paper is hung in an oven at 230°C. for four min. Alternately the paper is held horizontally over a hot plate until the lipid spots char (as the SO_3 fumes appear). These papers still contain enough sulfuric acid to be corrosive, but may be stored conveniently in Saran-Wrap.®

Quantitative Chromatographic Technique. Swartwout *et al.* (24), by measuring the absorbance of the charred spots with a photometer designed for the purpose, developed quantitative micro assay procedures for cholesterol and a number of other steroidal compounds. Also, they pointed out the general applicability of quantitative glass paper chromatography to a wide variety of organic compounds.

A set of standards is applied to each silicate-coated glass paper; e.g., 10 μl volumes of three standard cholesterol solutions (containing 3.0, 9.0 and 15.0 mg of cholesterol per 100 ml) are pipetted onto the paper, giving spots which contain 0.3 μg, 0.9 μg and 1. 5 μg of cholesterol, respectively. Suitable volumes of the solutions to be analyzed are also applied, so that the amount of cholesterol applied to each spot on the chromatogram lies between 0.2 μg and 1.5 μg. After chromatography and development of the charred spots in an oven, as described above, the glass paper is cut into pieces so as to permit densitometry of each spot of standard and of each unknown sample. The absorbance of each charred spot is read and recorded, as well as the absorbance of a glass paper blank cut out at the level of the spots. Then these pieces of glass paper, properly identified, are freed of the carbon char by hanging in a furnace at 600°C. for 15 min. The absorbance of the carbon residue in a given sample is obtained from the following relationship:

$A_s = (A_1 - A_2) - (A_3 - A_4)$

A_s = Absorbance of sample.
A_1 = Absorbance of glass paper with charred spot.
A_2 = Absorbance of glass paper after removing charred spot.
A_3 = Absorbance of glass paper blank.
A_4 = Absorbance of glass paper blank after removing the char of the reagent.

The absorbance of the standards is plotted on linear graph paper. The amount of material in the unknown samples is read from this graph. Two samples of pooled serum were analyzed by this method and by the method of Sobel and Mayer (25). The results are shown in Table I.

TABLE I

Comparison of Total Cholesterol Concentration of Two Pooled Sera by Glass Paper Chromatography and by Spectrophotometry.

Glass paper chromatography	Sobel-Mayer method
187 ± 9.3 mg./100 ml.[a]	179 mg./100 ml.
496 ± 21.1 mg./100 ml.	474 mg./100 ml.

[a] Av. Value ± S. D. (50 determinations).

The non-uniformity of the glass paper is the only reason for rereading the cut-out spots after removal of the char at 600°C. Should a sufficiently uniform paper become available, the labor involved in photometry would be halved, or work output for the same effort would be doubled.

Earlier glass papers from the Hurlbut Paper Co. required pre-treatment with solvent or a furnace at 600°C. to remove residual organic binder. Later papers from the same source and bearing the same designation, No. 934-AH, do not require this preliminary cleaning unless they become dirtied due to careless handling. It has become a practice with the authors, however, to pre-treat each paper at 600°C., for 15 min. in a furnace.[3]

Standard curves for a number of sterols, steroids, and bile acids were published by Swartwout *et al.* (24), and of phosphatidyl choline and sphingomyelin by Muldrey *et al.* (20) who also reported analyses of the various phosphatides in human blood and serum (20, 26). Preliminary results have been reported of the quantitative glass paper chromatography of human fecal sterols (27) and of cholesteryl esters and methyl esters of the higher fatty acids (28). Preliminary application of the quantitative assay to the cholesteryl esters of human maternal and cord sera revealed a serum cholesteryl ester fatty acid (CEFA) pattern characteristic of the adult of the species and characteristic of the newborn (29). Bowers *et al.* (30) applied this technique to the sera of patients with various liver diseases and observed an abnormal CEFA pattern in many such cases. The effect of nicotinic acid therapy on the human serum CEFA pattern has also been determined by this method (31). It is evident that this technique of quantitative glass paper chromatography can be applied successfully in the microgram range to a wide variety or organic compounds which can be made to separate chromatographically and which can be charred with sulfuric acid and heat.

Neutral Lipids. In their original report of impregnated glass paper chromatography, Dieckert and Reiser (12) described the separation of cholesteryl palmitate, cholesterol, tripalmitin, dipalmitin and monopalmitin. This technique has been used by Reiser *et al.* (32, 33) for following the progress of the separation of neutral lipids and phosphatides on silicic acid columns. Ory, Bickford, and Dieckert (34) were able to separate the methyl esters of brominated fatty acids on silicic acid glass paper. During

[3] A reliable well-thermostated 12 x 12 x 12 in. or 18 x 18 x 18 in. furnace may be obtained from the Blue Diamond Kiln Company, 1709 N. Hullen Drive, Metairie, Louisiana.

chromatography of serum lipids on silicic acid glass papers, it was observed that occasionally some fractionation of the cholesteryl esters was obtained which seemed to resemble the separations obtained by Klein and Janssen (35) on silicic acid columns. Efforts to improve the silicic acid impregnated glass paper for the purpose of separating the cholesteryl esters led to the development of a technique of impregnating the paper with silica gel. This paper gives a reproducible separation of cholesteryl esters as shown in Fig. 1. The primary effect observed depends on the number of double bonds present in the molecule. This same separation is obtained with the methyl esters of fatty acids, Fig. 2. This same separation of methyl esters of fatty acids may be obtained on silicic acid columns.

It was observed that on all of the chromatograms in which cholesteryl esters were separated from a serum extract on silica gel paper that free cholesterol moves to an R_f of about 0.1 when developed in isooctane while triglycerides are still at the origin. The addition of a more polar solvent to the isooctane causes an increase in the R_f of triglyceride which is greater than the concurrent increase in the R_f of cholesterol, so that in all solvents which we have investigated the relative positions become reversed and triglycerides reach the solvent front before cholesterol. The R_f value at which cholesterol and triglyceride reverse positions varies greatly and depends upon the polar solvent used. The addition of isopropyl acetate to isooctane causes the reversal to occur at such a low R_f value that a separation of triglyceride and cholesterol with the latter having a higher R_f value is not feasible. With 1,2-dichloroethane-isooctane mixtures, however, this reversal takes place at higher R_f values and a separation of cholesterol from triglycerides, without reversal, is readily obtained. An example of the use of these two solvent systems for the separation of cholesterol and triglycerides is shown in Fig. 3. These two solvent systems should prove to be useful for two dimensional chromatography for identification purposes. That this reversal probably also takes place on silicic acid columns can be deduced from the work of Hirsch and Ahrens (36) who studied the adsorption isotherms of lipids in a number of solvent systems.

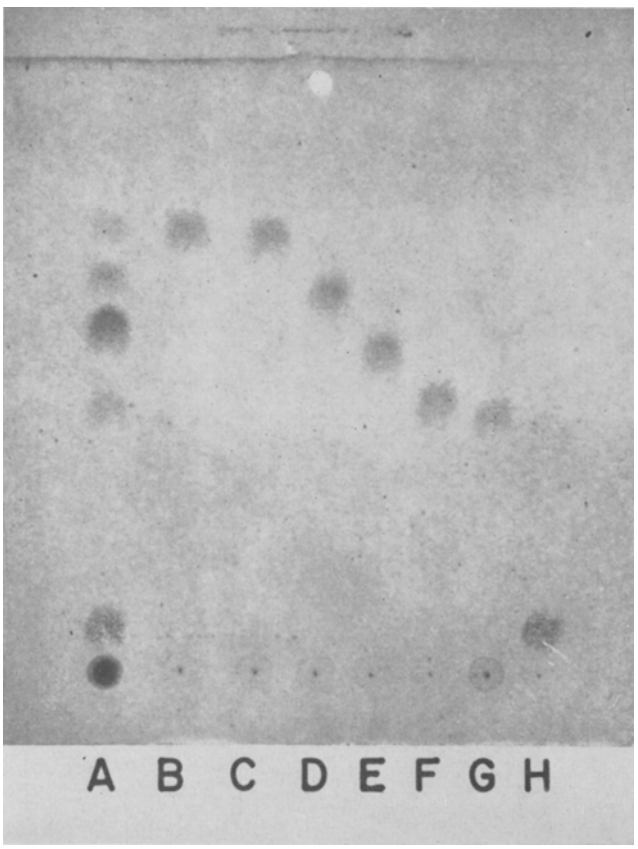

FIG. 1. Photograph of a chomatogram showing the separation of cholesteryl esters on "silica gel" glass paper. Developing solution: Isooctane. A. Alcohol-ether extract of serum. B. Cholesteryl stearate. C. Cholesteryl palmitate. D. Cholesteryl oleate. E. Cholesteryl linoleate. F. Cholesteryl linolenate. G. Cholesteryl arachidonate. H. Cholesterol.

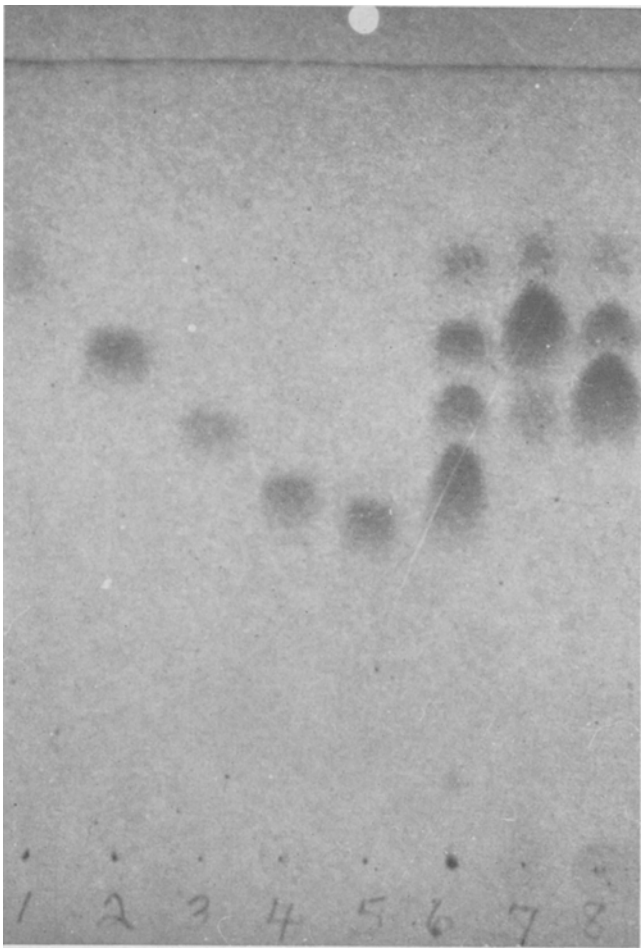

FIG. 2. Photograph of a chromatogram showing the separation of methyl esters on "silica gel" paper. Developing solution: Isooctane. 1. Methyl stearate and palmitate. 2. Methyl oleate, 3. Methyl linoleate. 4. Methyl linolenate. 5. Methyl arachidonate. 6. Mixture of 1, 2, 3, 4, and 5. 7. Methyl esters of vegetable shortening. 8. Methyl esters of cottonseed oil.

No useful fractionation of a natural mixture of triglycerides has been obtained, although tristearin and triolein can be separated from one another.

There is an effect of fatty acid chain length on adsorption as shown by Ory (37) who separated acetoglycerides on silicic acid glass paper, but the separability is less than that obtained by reverse phase paper chromatography (38).

Miscellaneous observtions on the chromatography of neutral lipids. Scattered observations which have been accumulated in the course of previous investiga-

tions but which have not been published previously are presented here with the hope that they may prove useful.

Glass paper may be used effectively as the support for a non-polar or "reversed" phase, in which case its properties are very similar to those of non-polar cellulose paper. Using glass paper coated with hydrocarbons and silicones, we have duplicated the separations of methyl esters of fatty acids, sterol esters, and coenzymes Q_9 from Q_{10} which have been reported on coated cellulose paper (38). However, the advantageous rapid solvent development usually characteristic of impregnated glass paper is lost, as is the other major advantage of the glass fiber paper: the sulfuric acid char reaction. The char technique is unusable with hydrocarbon coatings and usable with difficulty in the case of silicone coated papers which are quite difficult to wet with the sulfuric acid spray. Investigation of reversed phase glass papers has been discontinued in the absence of any definite advantage over reversed phase cellulose paper.

Coenzyme Q_9 and Q_{10} run as a single spot on silica gel glass paper with an R_f value just below that of cholesterol. We have been able to separate them still further from cholesterol by converting them to the more polar hydroquinones on the glass paper by overspotting with sodium borohydride solution prior to chromatography.

Since we use corn and cottonseed oil in experimental diets, we were prompted to attempt the determination of the sitosterol content of these oils by quantitative glass paper chromatography. This appeared to be relatively simple in view of the success of this technique when applied to cholesterol (24), since β-sitosterol has the same R_f as cholesterol on potassium silicate glass paper. A compound having an R_f value higher than β-sitosterol was observed in both corn and cottonseed oils and on further investigation was found in peanut and olive oils. In corn oil its concentration is almost as high as that of the material in the spot containing β-sitosterol, and it is a major component of the non-saponifiable fraction in all four oils. It was shown to be neither α-tocopherol nor γ-sitosterol (the latter, a gift of Dr. Arnis Kucsis). Our values for the sitosterol content of corn oil were approximately 300 mg per 100 ml as compared with 800 mg per 100 ml, the value listed by the manufacturer. (We were unable to analyze samples which were also analyzed by the manufacturer, who had discontinued sitosterol determinations on their oils.)

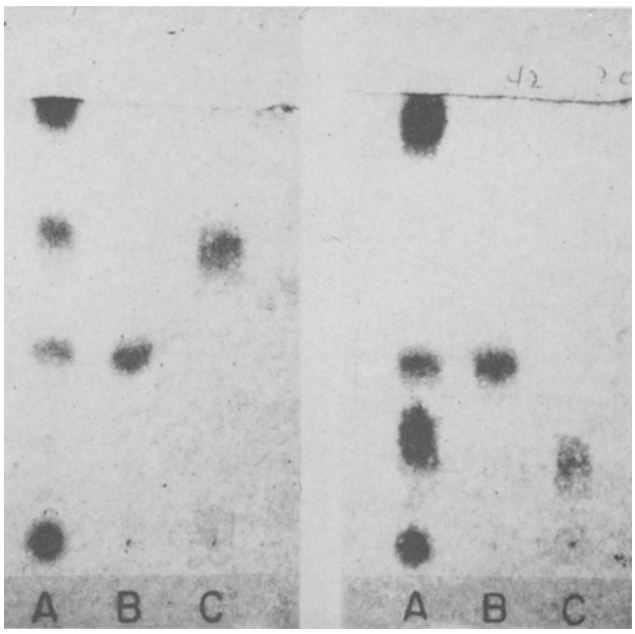

FIG. 3. Photograph of a chromatogram showing the separation of cholesterol and triglycerides on "silica gel" glass paper. Developing solution: Chromatogram on Left-Isooctane-Isopropyl acetate-100–2, Chromatogram on Right-Isooctane 1,2-dichloroethane 100–30. A. Alcohol-ether extract of serum. B. Cholesterol. C. Triglycerides (cottonseed oil).

Alpha tocopherol, which has a much higher R_f value than does cholesterol or sitosterol, may also be chromatographed in its more stable and more polar oxidized form by adding ferric chloride before chromatography. Glass paper chromatography might well be adapted to the quantitative determination of α-tocopherol.

Squalene, the C_{30} isoprenoid precursor of cholesterol, travels with the solvent front on silica gel paper with isooctane as the developing solvent.

Carotenoid and chlorophyll pigments are rapidly separated on silica gel glass paper. The separations are similar to those on columns, and to separations reported by Strain on glass paper. Chromatography of plant pigments has been used entirely for purposes of demonstration since the colors permit continual observation of the progress of development of the chromatograms. The pigments are rapidly destroyed by the gel paper, but the separations are rapid enough to be useful for qualitative assay of column-fractions. It is possible that some adsorbent other than silica gel might cause less destruction and prove more useful for these compounds.

Phospholipids. Four chromatographic systems for phosphatide separations on glass paper have been reported. The first by Dieckert and Reiser (16), employed silicic acid impregnated glass paper and methanol-ethyl ether as solvent. Although it represented an important advance in chromatography at the time, this system produced elongated spots. Improved separations were obtained when the acetone-ether-phenol-water solvent of Dieckert and Morris (39) was applied to the chromatography of phospholipids on silicic acid impregnated glass paper (40). The third system to employ silicic acid impregnated glass paper was described by Agranoff, Bradley and Brady (41), who reported that phosphatide separations on silicic acid impregnated glass papers with the diissobutylketone-acetic acid-water system were very similar to separations obtained with the same solvent system on silicic acid-coated cellulose paper. On silicic acid glass paper in these three solvent systems the phospholipids separate as follows, in order of decreasing R_f values: the cephalins, phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS), lecithin or phosphatidyl choline (PC) and sphingomyelin (Sp). The lysophosphatides have R_f values smaller than the corresponding parent compounds and often overlap other phosphatides of smaller R_f value than the parent compounds. For example, lyso-PE may occupy the same spot as PC, etc.

The fourth system for the chromatography of phosphatides on glass paper, described by Muldrey, Miller and Hamilton (20, 26), differs fundamentally from the other three systems. A major reversal of the migration order (relative R_f values) is effected by means of an alkaline silicate coating of the glass paper and

a benzene-pyridine-water (BPW) solvent. In this system the aminophosphatides, PE and PS and their lyso compounds, have R_f values smaller than those of PC and SP. On glass papers coated with 0.4% (w/v) aqueous sodium silicate solution, free fatty acids (FFA), PC, Sp and PE can be separated cleanly, both in model mixtures and in extracts of natural mixtures, e.g., lipid extracts of blood, serum, liver and brain. The neutral lipids in natural mixtures are removed to the solvent front in a seven min. "benzene sweep" and, therefore, do not interfere with the subsequent chromatography of the phosphatides. Satisfactory separations of PS from natural lipid mixtures are obtained on a more heavily impregnated (2% silicate solution) glass paper. The principle of the benzene sweep is extended to a preliminary short development in a second solvent, BPW 100:125:13, which leaves PS at the origin, but moves PC, Sp and PE up the paper; subsequent development in BPW 100:125:25 moves PS as a compact separate spot. The second and third solvents may be applied with an intervening drying in vacuum. In an alternate procedure which is tantamount to gradient elution, the second solvent, BPW 100:125:13 is permitted to travel about half-way up the paper, and the paper is then transferred directly to the third solvent, BPW 100:125:25. Fig. 4 illustrates a chromatogram obtained with this technique. Standard curves for quantitative glass paper chromatography of these phosphatides are shown in Fig. 5. Some preliminary results of the quantitative determination of blood and serum phosphatides by this procedure are shown in Table II.

TABLE II

Mg. of Lipid Per 100 ml. of Serum or Blood

		PC	Sp. and Lyso PC	PE	PS
Maternal serium	(J.D.)	132	65	12	0
Cord blood serum	(J.D.)	40	36	5.2	1.6
Maternal serum	(M.F.)	117	76	3.0	2.0
Cord blood serum (marked hemolysis)	(M.F.)	26	35	6.0	3.2
Fasting adult male	(E.M.)	164	57	8.2	0
serum		156	109	36	25
blood					
Fasting adult male	(E.M.)	151	63	6.0	4.4
serum		147	97	33	21
blood					

It should be pointed out that PC, PE, PS, lyso PE and lyso PS are resolved as single spots in this system, but Sp and lyso PC appear at the same R_f value. Also noted was the appearance of plasmalogen lipid at the R_f level of its diglyceride analog: phosphatidal choline with phosphatidyl choline, phosphatidal ethanolamine and serine with PE and PS, respectively. Serum lipid extracts contain a small amount of plasmalogen material in the Sp spot; this would seem to be lysophosphatidal choline.

The sodium and potassium silicate impregnated glass papers give separations of PE and PS which are better than those of the three silicic acid systems described. Rouser *et al* (42) employed the same principle when they separated on a silicic acid- ammonium silicate column the PE and PS which are eluted together from silicic acid columns.

The pyridine solvent system has proved interesting when applied to cerebrosides and other brain lipids (43) and has been adapted to silicic acid cellulose papers by Hack (44) and coworkers (45) for the separation of cerebrosides and sulfatides.

Steroids, Bile Acids, Sterols and Sapogenins. Impregnated glass fiber paper has proved to be particularly useful for the separation of polycyclic lipids. In an investigation designed primarily to gather information about the effect of various functional groups and isomers on adsorbability Hamilton and Dieckert (46) studied a number of steroids. Two different pairs of cis-trans isomers were readily separated, allopregnane-3β-ol-20-one from pregnane-3β-ol-20-one (5α and 5β) and estradiol-17β from estradiol-17α. Three pairs of alcohols and their corresponding ketones were also separated, cortisone from hydrocortisone, 11-dehydrocorticosterone from corticosterone, and estrone from both estradiols. Although the position isomers, corticosterone and 17α-hydroxy-11-deoxy-corticosterone were not separated in the simple solvent systems and short papers used in this investigation, they were later separated by Dingman, *e al* (47). The same general conclusions held also for the bile acids (22). Three of the four isomers of 3α, 6(α or β), 7(α or β)-trihydroxycholanic acids were found to be readily separable. More recently the fourth isomer (hyocholic acid) became available and was also found to be separable from the other three (43). These compounds include both cis-trans and position isomers. Deoxycholic acid was not separable from chenodeoxycholic acid on sodium silicate, silicic acid, phosphate or alumina paper, but has more re-

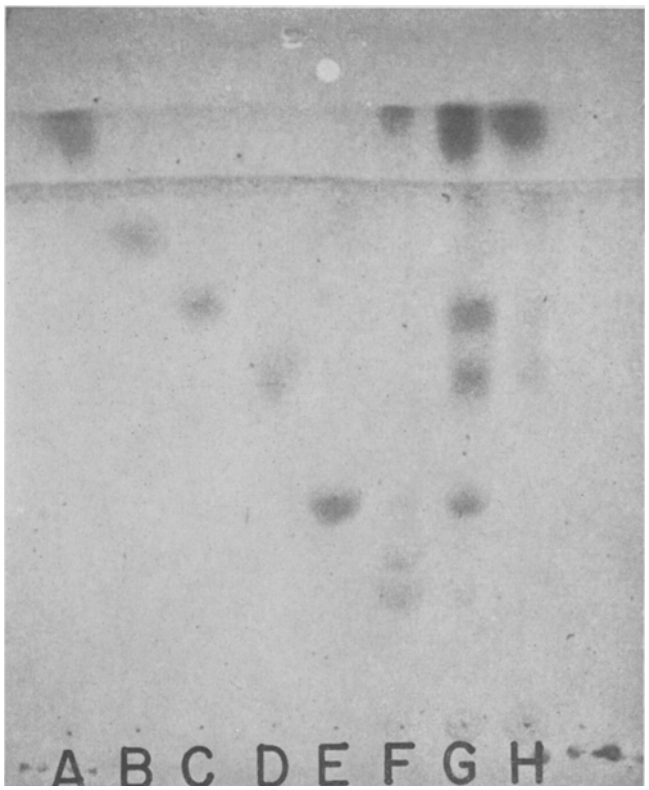

FIG. 4. Photograph of a chromatogram showing the separation of phosphatides on glass paper impregnated with 2% sodium silicate solution. Developing solvents: First, benzene, developed to upper front (14 cm.). Second, benzene-pyridine-water (BPW 100-125-13, developed until solvent traveled 5 cm., then transferred directly to third solvent. Third, BPW 100:125:25 until solvent rose to lower front (12 cm.). A. Triglyceride and cholesterol. B. Fatty acid. C. Phosphatidyl choline. D. Sphingomyelin. E. Phosphatidyl ethanolamine (PE) F. Mixture containing (in order of decreasing Rf) neutral lipid, PE, lyso-PE and phosphatadyl serine. G. A chloroform-methanol extract of blood serum.

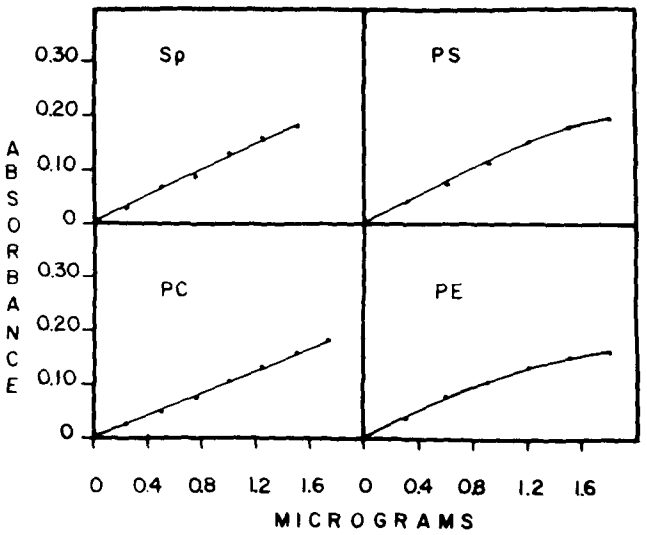

FIG. 5. Standard curves of phosphatidyl choline (PC), sphingomyelin (Sp), phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS). The absorbance of the charred spot is plotted against the amount of phosphatide applied to the chromatogram.

cently been separated on phosphate paper in a different solvent (43). The ability to run a large number of chromatograms in a short period of time makes it feasible to test a large number of solvent systems. The different papers also have different chromatographic properties. This is illustrated in Table III for lithocholic acid, 3,12-diketocholanic acid and dehydrocholic acid in benzene-ethanol as a chromatographic solvent. 3,12-diketocholanic acid has a higher R_f value than does dehydrocholic acid on all three papers while lithocholic acid occupies all three possible positions with respect to these two.

The isolation of sapogenins of the peanut by Dieckert, Morris and Mason (19) is an excellent example of the use of impregnated glass paper as a tool for the isolation and identification of natural products. They were able to separate the sapogenins into three distinct fractions on a silicic acid column. Impregnated glass paper was used as an assay to follow the progress of this column. Glass paper chromatography also made it obvious that none of the three fractions was homogeneous and that they could not be further resolved by silicic acid chromatography.

The resolution of these mixtures was accomplished by use of a glass paper column and again the progress of the fractionation was followed by glass paper chromatography. The value of the different adsorbents for impregnating glass fiber paper was illustrated, e.g., peanut sapogenins A_1 and A_2 were separable on glass paper treated with phosphate but not on silicic acid paper while for soya sapogenols B and D the reverse was true. They were able to isolate four different peanut sapogenins by this technique and show

TABLE III

R_f Values of Bile Acids on Three Different Impregnated Glass Papers

	A	B	C
3, 12-diketocholanic	0.54	0.67	0.35
Dehydrocholic	0.47	0.46	0.25
Lithocholic	0.36	0.55	0.68

A. Potassium silicate paper, Benzene-ethanol 100:5.
B. Phosphate paper, Benzene-ethanol 100:1.
C. Silicic acid paper, Benzene-ethanol 200:1.

that one of them was probably identical to soya sapogenol B. It was conclusively shown by cochromatography on impregnated glass fiber paper that the other three peanut sapogenins isolated were not identical to soya sapogenols A, C, and D.

A preliminary report of the use of glass paper chromatography for the separation of fecal sterols was reported in 1959 (27). The recent finding that silica gel glass paper could be used for the separation of cholesteryl esters led us to investigate the possibility of separating sterols based on the number of double bonds. Other sterols were included with the idea of using this technique for investigating fecal sterols and for use in the study of sterol biosynthesis (48).

Two pairs of sterols differing only in the position of double bonds were found to be separable after acetylation, cholesterol (Δ^5) from Δ^7-cholestenol and desmosterol ($\Delta^{5,24}$) from zymosterol ($\Delta^{8,24}$). Cholesteryl acetate and desmosteryl acetate were separable; the later, containing one additional double bond, had the lower R_f. None of these compounds were separable as free sterols. Lanosterol ($\Delta^{8,24}$-4,4,14-trimethylcholestadienol), methostenol (Δ^7-4α-methylcholestenol) and cholesterol were separable as free sterols, but not as acetates.

Alpha and β-cholestanol were readily separable whereas α and β-coprostanol were separable only with difficulty. Cholestanone and coprostanone were not separable from each other but were completely separated from sterols.

Glass paper chromatography has been developed as a method for the determination of urinary aldosterone by Staub and Dingman (49).

Discussion

Glass fiber paper was selected as a support medium for silicic acid because of its inertness to high temperature and corrosive reagents. From its inception by Dieckert it was operated as if it were an adsorption chromatogram. The simplicity of adsorption chromatography compared with liquid-liquid partition and the vast literature on adsorption chromatography has led us, also, to prefer to use it as if it were an adsorption chromatogram. In the case of more polar compounds, such as phosphatides, there is some doubt about the nature of the stationary phase which allows one to separate these compounds, but selection of solvent systems using adsorption chromatography theory has proved most useful.

A deliberate effort has been made to use lighter impregnation of adsorbent, e.g., 1 to 2% solution as contrasted to the usual 10 to 20%, which increases the sensitivity of the char formation and increases the separation factor between compounds. This is similar to the findings of Stahl (11) for thin layer chromatography. The lightly impregnated papers are much simpler to prepare since no precautions are required to coat the papers evenly. Complicated washing procedures have been completely eliminated for all of the impregnated papers except silicic acid. The volatility of ammonium chloride has proved to be of particular advantage for the formation of alumina and the new "silica gel" paper. If an adequate muffle furnace is not available the ammonium chloride may be removed by washing with water. In our laboratory the use of "silica gel" paper has largely replaced the use of silicic acid paper. The lightly coated papers have the

one disadvantage of low capacity. In general, from 0.2 to 2.0 micrograms of each lipid are separable as symmetrical spots. This gives an adequate working concentration range, conserves lipid material, and is only disadvantageouus when it becomes desirable to isolate material from the chromatogram or use a technique for locating the compounds which is less sensitive than the sulfuric acid char reaction.

Another major advantage of impregnated glass paper, besides inertness, is the rapidity with which solvents develop on the paper. Often a 10 cm development (approximately seven min.) and rarely more than 20 cm (20 min.) gives the desired separation.

The third major advantage, the quantitative densitometry of the char, offers the possibility of quantitative determination of a wide variety of organic compounds which might be difficult to assay by other methods.

Impregnated glass fiber paper has a high discriminatory power for changes in functional groups, differences in unsaturation, and isomerism, but is less useful than "reversed phase" for separations based on differences of molecular size. For example, the additional 5 carbons in Q_{10} over Q_9 is almost exactly balanced by the addition of one double bond.

For the past two years glass paper chromatography has been used successfully for teaching lipids in the freshman medical biochemistry laboratory. During an experiment in which the students prepare egg albumin and lecithin by conventional methods, the chromatography of phosphatides and neutral lipids of both egg yolk and blood serum are demonstrated. Later in the course the students are required to estimate the cholesterol and cholesteryl esters in their own serum. The students are required to do all of the manipulations with the exception of preparing the silica gel paper. Three serum extracts from a high normal, a low normal, and a cirrhotic serum are provided as reference standards. The concentration of cholesterol and of each cholesteryl ester in each of the reference extracts is made known to the students. Each student prepares an 11X extract by pipetting 0.5 ml of his serum into 10 ml of ethanol-isopropyl ether (2:1 v/v), centrifuges and prepares a 22X extract by a two-fold dilution with ethanol-isopropyl ether. The two unknown extracts are spotted between the standards, the chromatograms are developed in isooctane in 800 ml beakers, air-dried, sprayed with sulfuric acid, and heated over a hot plate. The student views the chromatogram by transmitted light and estimates the amount of each cholesteryl ester by comparison with the spots from the standard solutions. The high student interest in this analysis is partially because he actually visualizes the cholesteryl ester fatty acid pattern of his own serum between the high and low normal sera and next to the cirrhotic serum, and partially because of the current interest in the relationship of serum cholesterol to atherosclerosis.

The separations of sugars on impregnated glass papers by Jayme and Knolle (50) and by Dieckert and Morris (39) and Gabriel and Igals (51) represent an extension of the technique toward partition chromatography. The mucopolysaccharide separations of Dalferes and Berenson (52) indicate the possibility of adapting glass paper methods to separation of polymeric materials of different molecular size. The recent report by Mutschler and Rochelmyer (53) of amino acid separations on thin layers suggests that similar separations could be accomplished on suitably impregnated glass papers.

This paper is presented primarily as the technique of glass paper chromatography as used in the authors' laboratories and is not intended as a critical review.

As a closing thought the authors would concur with the opinion of Horáček and Černíková (54) that "glass paper chromatography deserves more extensive application."

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