16. Cannon, J.A., Zilch, K.T., and Dutton, H.J., Anal. Chem., *24*, 1530 (1952).
17. Scholfield, C.R., Nowakowska, J., and Dutton, H.J., J. Am. Oil Chemists' Soc., *37*, 27 (1960).
18. Nichols, P.L., Jr., J. Am. Chem. Soc., *74*, 1091 (1952).
19. Dutton, H.J., and Cannon, J.A., J. Am. Oil Chemists' Soc., *33*, 46 (1956).
20. Scholfield, C.R., and Hicks, M.A., J. Am. Oil Chemists' Soc., *34*, 77 (1957).
21. Scholfield, C.R., and Dutton, H.J., J. Am. Oil Chemists' Soc., *35*, 493 (1958).
22. Scholfield, C.R., Nowakowska, J., and Dutton, H.J., J. Am. Oil Chemists' Soc., *38*, 175 (1961).
23. Scholfield, C.R., and Dutton, H.J., J. Am. Oil Chemists' Soc., *36*, 325 (1959).
24. Dutton, H.J., Scholfield, C.R., and Mounts, T.L., J. Am. Oil Chemists' Soc., *38*, 96 (1961).
25. Mattson, F.H., and Lutton, E.S., J. Biol. Chem., *233*, 868 (1958).
26. Kurht, N.H., Welch, E.A., Blum, W.P., Perry, E.S., and Weber, W.H., J. Am. Oil Chemists' Soc., *29*, 261 (1952).
27. Mattson, F.H., Benedict, J.H., Martin, J.B., and Beck, L.W., J. Nutrition, *48*, 335 (1952).
28. Kurht, N.H., Welch, E.A., Blum, W.P., Perry, E.S., Weber, W.H., and Nasset, E.S., J. Am. Oil Chemists' Soc., *29*, 271 (1952).
29. Perry, E.S., and Brokow, G.Y., J. Am. Oil Chemists' Soc., *32*, 191 (1955).
30. Fugger, J., Zilch, K.T., Cannon, J.A., and Dutton, H.J., J. Am. Chem. Soc., *73*, 2861 (1951).
31. Cannon, J.A., Zilch, K.T., Burket, S.C., and Dutton, H.J., *J. Am. Oil Chemists' Soc., 29*, 447 (1952).
32. Johnston, A.E., Zilch, K.T., Selke, E., and Dutton, H.J., J. Am. Oil Chemists' Soc. In Press.
33. Fugger, J., Cannon, J.A., Zilch, K.T., and Dutton, H.J., J. Am. Oil Chemists' Soc., *28*, 285 (1951).
34. Frankel, E.N., Evans, C.D., McConnell, D.G., Selke, E., and Dutton, H.J., J. Org. Chem., *38*, 367 (1961).
35. Scholfield, C.R., Dutton, H.J., Tanner, F.W., Jr., and Cowan, J.C., J. Am. Oil Chemists' Soc., *25*, 368 (1948).
36. Scholfield, C.R., McGuire, T.A., and Dutton, H.J., J. Am. Oil Chemists' Soc., *27*, 352 (1950).
37. McGuire, T.A., and Earle, F.R., J. Am. Oil Chemists' Soc., *28*, 328 (1951).
38. Carter, H.E., Celmer, W.D., Galanos, D.S., Gigg, R.H., Lands, W.E.M., Law, J.H., Mueller, K.L., Nakazama, T., Tomizawa, H.H., and Weber, E., J. Am. Oil Chemists' Soc., *35*, 335 (1958).
39. Mason, L.H., and Johnston, A.E., Cereal Chem., *35*, 435 (1958).
40. Cole, P.G., Lathe, G.H., and Ruthven, C.R.J., Biochem. J., *54*, 449 (1953).
41. Olley, J., Chem. & Ind. (London), 1120 (1956).
42. Lancaster, C.R., Lancaster, E.B., and Dutton, H.J., J. Am. Oil Chemists' Soc., *27*, 386 (1950).
43. Curl, A.L., J. Agr. Food Chem., *1*, 456 (1953).
44. Burnett, M.C., Lohmar, R.L., and Dutton, H.J., J. Agr. Food Chem., *6*, 374 (1958).

Paper Chromatography of Lipids: Methods, Applications, and Interpretations

GEORGE ROUSER, A.J. BAUMAN, NICHOLAS NICOLAIDES,[1] and DOROTHY HELLER,
Department of Biochemistry, City of Hope Medical Center, Duarte, California

THIS REPORT describes methods for the paper chromatography of lipids and shows some of the ways the methods can be applied. The proper use of paper chromatography requires a clear understanding of the limitations of the method, and the limitations and uses are best appreciated against a background of interpretations concerning the mechanism of the chromatographic process at the molecular level. An effort is made to describe the probable basis for paper chromatographic separations in this article, and for column chromatographic methods in another report.

Paper chromatography is useful for the comparisons of compounds as an aid in identification, for the detection and tentative identification of impurities, reaction products and intermediates, and for the exploration of variables before undertaking column chromatography. The apparatus is simple and the process is not difficult. A complete and reliable characterization of a lipid by paper chromatography is not always possible. Even when compounds are compared with different types of solvent systems, one should consider the results tentative until confirmed (spectral properties, hydrolysis products, formation of derivatives). Paper chromatography has set new, high standards for the purity of lipids and allied substances, and has been used extensively in the development of new column procedures and for monitoring of column fractions.

Paper chromatography is not generally as useful as column chromatography for the isolation of pure lipids. The ease with which pure substances can be isolated in large amounts suitable for complete characterization makes column procedures more desirable. Exposure to air and autoxidation are easily avoided in column chromatography.

Attempts to use paper chromatography for quantitative determinations are generally not entirely satisfactory. Fairly precise quantitative paper chromatographic methods can be developed for authentic compounds. When the methods are applied to biological samples however a number of difficulties are encountered. A single spot on a chromatogram may represent more than one substance. Another difficulty is the selection of a sensitive, specific assay method for small amounts of material. Color reactions are difficult to control and the sensitivity may vary. When an elution method is used, impurities eluted from the paper along with the sample may influence the determinations. Paper chromatography is sometimes selected for quantitative determinations because of the mistaken impression that column chromatographic procedures can not be made to handle extremely small samples. Although the ease with which small samples may be examined by column chromatography does vary, the extremely small amounts of lipids that can be detected and determined by gas chromatography make it quite clear that column chromatography can be even more sensitive than paper chromatography, or modifications such as thin-layer chromatography.

Types of Paper Chromatography

The chromatographic process is recognized as being one of adsorption or partition. Adsorption columns have been used widely, but the process is seldom used

[1] On leave of absence from the Department of Biochemistry and Division of Dermatology, University of Oregon Medical School, Portland, Oregon.

for paper chromatography. Adsorption chromatography may be distinguished readily from partition chromatography at the molecular level. In adsorption chromatography the substance being chromatographed attaches directly to the solid adsorbent, that is, the strictly immobile part of the stationary phase. In partition chromatography the substance being chromatographed does not attach directly to the completely immobile part of the stationary phase, but rather attaches through liquid (solvent) molecules to the insoluble (immobile) part of the stationary phase. This attachment in partition chromatography is generally through water molecules; thus hydrogen bonding plays a major role in partition chromatography.

Ion exchange chromatography may meet the definition for adsorption chromatography, but ion exchange is conveniently considered as a separate process because column performance is different and in many cases is more like partition chromatography. This may be related to the fact that ions may be hydrated, etc., and not show simple behavior.

Adsorption can be distinguished from partition by the results obtained by column or paper chromatography. Adsorption chromatography is characterized by long tails on peaks. Similarly elongated spots are to be expected on paper chromatograms. This characteristic limits the value of adsorption for chromatography. The tailing is brought about by the fact that relatively more substance is adsorbed per gram of adsorbent from more dilute solutions, i.e., as the concentration of a substance in the external solution decreases, the ratio of amount of substance adsorbed to the amount remaining in solution increases. As one molecule of substance undergoing adsorption may interfere with the adsorption of another molecule, the greater efficiency of adsorption at low concentration is readily appreciated. Tailing is not an invariable feature of partion chromatography because in this process attachment to the stationary phase is not as firm and, as attachment is through solvent molecules usually present in large excess, the process remains constant despite changes in concentration of the substance(s) being chromatographed. The detachment of the substance from the stationary phase in partition chromatography is accomplished by breaking hydrogen bonds and other noncovalent bonds between the substance being chromatographed and fairly mobile solvent molecules that make up a portion of the stationary phase.

It is to be emphasized that adsorption and partition chromatography can be distinguished readily by examination of the column or paper chromatographic results. The fact that the process on paper chromatograms and thin-layer chromatograms (stationary phase spread on a glass plate) is one of partition for the most part rather than adsorption can be seen immediately from the fact that discrete spots are obtained without trailing or streaking. This emphasizes the partition chromatographic nature of these systems. Ion exchange is also involved in many systems. The thin-layer technique is thought of by some as an adsorption process, but the reasons for this are not clear. The discreteness of the spots obtained, the inevitable presence of water in the system (despite activation by heat because of reabsorption of water from the atmosphere during spotting to plates, etc.), and the fact that water is required to obtain good round spots, all indicate that the process is partition rather than adsorption chromatography.

Paper and column chromatographic procedures are frequently designated as reverse phase chromatographic systems. Paper chromatography was initially applied to polar and water soluble substances where the stationary phase is composed primarily of cellulose and bound water. Water molecules hydrogen bond to the hydroxyl and other groups of the cellulose to form the "stationary" phase. A less polar mobile phase migrates down the paper. When this system is reversed, that is, when a nonpolar substance is present in the stationary or nonmobile phase and a more polar solvent migrates down the paper, the procedure may be termed reversed phase chromatography. Reversed phase systems are obtained by impregnating paper with substances such as paraffin oil or silicone oil. The polar functional groups of the paper are no longer important components of the stationary phase, but rather paper becomes simply a support. Similar techniques can be used for column chromatographic work.

It is to be clearly understood that the impregnation of paper with silicic acid, various silicates, and other polar substances does not provide a reversed phase system. These polar substances interact with the polar groups of the lipid molecules in contrast to the reverse phase systems (such as hydrocarbon stationary phases) that interact with the hydrocarbon portion of the lipid molecule. While silicic acid and other similar polar stationary phases fractionate lipids on the basis of the polar groups, the reverse phase systems fractionate lipids on the basis of length and unsaturation of the hydrocarbon chains. With polar stationary phases, such as silicic acid, there is generally a relatively small amount of fractionation of lipids on the basis of fatty acid composition (chain length and unsaturation) so that an entire lipid class may be obtained as a single spot on a chromatogram or as a relatively uniform peak on a column regardless of fatty acid composition differences. On the other hand a reverse phase system is of limited use for the fractionation of substances on the basis of polar groups as relatively small changes in polarity radically alter migration so that substances differing by one polar group migrate so far apart that different classes of lipids may not be seen in the body of the chromatogram.

It should be appreciated however that polar stationary phases do not always show exact correspondence of lipids of the same class but with different fatty acid compositions. It is usually observed that a peak from a column chromatogram is not uniform in its fatty acid composition when small subfractions are examined. There is some fractionation according to fatty acid composition. The same situation holds for paper chromatography (see Figs. 18 and 19). Slight differences in migration are difficult to detect, but when large differences in chain length are involved, as for example with cholesterol palmitate and cholesterol acetate, the two substances differing in chain length can be separated completely. Other more polar substances do not show this effect to the same extent.

Reverse Phase Paper Chromatography

Nonpolar Stationary Phase. We do not intend to consider reverse phase paper chromatography in detail in the present discussion because, despite the value of some of the methods, gas chromatographic

procedures can largely supplant them. Reverse phase paper chromatography has been used for a number of years for the higher fatty acids. Following the initial observations of Kaufmann and Nitsch (1), who used paraffin oil or undecane impregnated paper and developed chromatograms with a mixture of acetic acid and water, a number of systems of a similar nature have been reported. These include the use of silicone oil as a reverse phase component (2) and modifications of the original Kaufmann and Nitsch procedure.

All of these systems have one major defect. So-called critical pairs of fatty acids are not separable. The critical pairs are oleic and palmitic acids, myristic and linoleic acids, etc. A carbon chain length decrease of two carbon atoms is generally equivalent to the increase in polarity of the molecule brought about by the presence of a *cis* double bond. It is of some interest to inquire as to the reason for this behavior.

The solubility of a fatty acid or other hydrocarbon chain in a hydrocarbon or hydrocarbon-like stationary phase depends upon the interaction of hydrocarbon chains of the stationary phase and of the fatty acids by van der Waals attractive forces. The extent of these interactions will increase with increasing chain length and should decrease with the introduction of a double bond.

With the reverse phase paper chromatographic system for fatty acids the presence of a *cis* or *trans* double bond is of considerable importance, and *cis* and *trans* isomers may be separated. The *trans* double bond produces a minimal shortening of the carbon chain in the extended form, and thus decreases the interaction with the saturated hydrocarbon phase to a minimum extent. It is therefore not surprising that elaidic acid migrates only slightly ahead of stearic acid in the reverse phase paper chromatographic system of Kaufmann and Nitsch. A *cis* double bond, on the other hand, reduces the effective chain length more and the extent to which molecules line up and attractive forces are established is decreased even more. Some differences should be expected between the *cis* and *trans* double bond, but it might also be expected that some separation could be obtained between oleic and palmitic acids. Since the polarity introduced into the oleic acid molecule from the double bond is important in increasing the solubility of the fatty acid in the polar mobile phase (dipole-dipole interactions), the unsaturated fatty acid migrates even further down the paper than one might have expected from a simple decrease in the retention of the molecule in the stationary phase.

It is instructive to compare reverse phase chromatography with the gas liquid chromatographic process where an inert mobile phase is utilized. The gas chromatographic process is different from the paper chromatographic process because the substance being chromatographed is dissociated from the stationary phase by heat rather than by solvent elution, and the substance being chromatographed is moved through the column with a flow of inert gas, i.e., the gas does not interact to any appreciable extent with the molecules being chromatographed. Because of the inertness of the mobile carrier gas in gas chromatography, the critical pairs that are encountered in reverse phase paper chromatography can be separated. It is of some interest to speculate upon the potential use of gas chromatography with a carrier gas of sufficient polarity to influence the separation process itself. This can be accomplished by using relatively low polarity gases or a mixture of an inert gas and a small amount of a more polar gas. Such systems should have different resolving powers.

Other valuable reverse phase systems for lipids have been developed in particular Kaufmann's laboratory. The methods for the separation of triglycerides (3) and of the dinitrophenylhydrazones of fatty aldehydes (4) are useful. It is to be noted that fatty aldehydes may be separated conveniently and quantitated by gas chromatography.

Polar Stationary Phase Chromatographic Methods

1. Reasons for the impregnation of filter paper, results with different agents, and the basis for the chromatographic separations.

Unimpregnated filter paper can be used for the separation of lipid materials with appropriate solvents (5). The major difficulties encountered with unimpregnated filter paper are a lack of reproducibility related to the difficulties encountered in controlling the water content of the system, and to the relatively low capacity of such systems. Virtually all of the separations that are achieved on unimpregnated filter paper are probably by means of hydrogen bonding interactions between the polar functional groups of the paper and the substance being chromatographed, probably through the mediation of water molecules.

Paper may be impregnated with an insoluble polar substance to obtain increased capacity. From the historical standpoint silicic acid was the first truly successful substance used to increase the capacity of a polar stationary phase system for chromatography of lipids. This account will not follow the historical development; instead results will be presented in a way that demonstrates the basis of the chromatographic process.

A variety of salts including sodium and potassium phosphates, sodium sulfate and carbonate, and even sodium chloride, may be used to impregnate filter paper for the chromatography of lipids (6,7). By varying the polarity of the solvent used as the mobile phase, it is possible to obtain separations of nonionic and relatively nonpolar lipids or ionic and highly polar lipids. The molecular interactions appear to be to a great extent those of hydrogen bonding and dipole-dipole interactions of the lipids through water to the polar groups of the stationary phase. That the hydrogen bonding process for the more polar components and dipole-dipole interactions for the less polar substances are of primary importance is indicated by the fact that similar separations can be obtained by using substances such as dimethylformamide. As an example, the system that we have described using dipotassium hydrogen phosphate for impregnation of filter paper with the development of the chromatogram with chloroform/methanol, 4/1, (v/v) can be reproduced closely with the same solvent mixture when dimethylformamide is used for impregnation of paper. Salts such as sodium chloride are less effective than salts such as sodium acetate that contain an oxygen functional group.

Water is an important part of the stationary phase in these systems. This can be demonstrated readily by carefully maintaining the system in an anhydrous or nearly anhydrous state. When the water content of

TABLE I

Paper Chromatographic Systems

Solvents	Conditions*	Compounds	Figures
(1) n-Hexane	1, 60 min.	Saturated and unsaturated hydrocarbons, fatty acid esters, sterol esters	1, 26
(2) Benzene	1, 90 min.	Fatty acids, sterols, sterol esters, mono-, di-, and triglycerides	2, 3
(3) 5% diethyl ether in n-hexane	1, 60 min.	Compounds in (2) and vitamin K, tocopherols (vitamins E), coenzyme Q, hydroxy acids, vitamins A and D_2	4–6, 23, 25, 27
(4) 10% diethyl ether in n-hexane	1, 60 min.	Compounds in (3) and the methyl esters of the 2- to 16-hydroxypalmitates and other hydroxy fatty acids	7–10
(5) 25% diethyl ether in n-hexane	1, 60 min.	Sterols, hydroxy fatty acids and their methyl esters, mono- and diglycerides, α-glyceryl ethers	22
(6) 40% diethyl ether in n-hexane	1, 60 min.	Substances with 2 hydroxyl groups or 1 hydroxyl and 1 carboxyl group	11, 12, 24, 28, 29
(7) 60% diethyl ether in n-hexane	1, 60 min.	As for (6)	.....
(8) 100% diethyl ether	1, 60 min.	Substances with 2 or 3 free hydroxyl groups or 2 hydroxyl groups and 1 carboxyl group (ceramides, cholic and deoxycholic acids, etc.)	.....
(9) $CHCl_3$/acetone, 4/1	1, 60 min.	Ceramides (compounds with 2 to 4 free hydroxyl groups)	.....
(10) $CHCl_3/CH_3OH$, 7/1	2 and 3, 30 min.	Phospholipids	20, 21
(11) $CHCl_3/CH_3OH$, 4/1	2 and 3, 30 min.	Phospholipids, sulfatides	.....
(12) $CHCl_3/CH_3OH$ (4/1 + 8 ml./l. conc. aqueous ammonia)	4, 120 min.	Phospholipids, cerebrosides, sulfatides	15–19, 30, 31, 32
(13) $CHCl_3$/acetone(4/1), 85 vols. + 90% formic acid, 15 vols	4, 120–180 min.	Particularly useful for sulfatides	.....
(14) (a) $CHCl_3$/acetone (4/1), 95 vols. + (b) glacial acetic acid/water (9/1), 5 vols	4, 180 min.	Ceramides, cerebrosides, some phospholipids	.....
(15) As for (14) but (a) and (b) mixed 85/15	4, 180 min.	Phospholipids, cerebrosides, sulfatides	13, 14
(16) As for (14) but (a) and (b) mixed 75/25	4, 180 min.	Phospholipids, sulfatides	.....
(17) n-Butanol/acetic acid/water (4/1/5) (upper layer)	5, 8–18 hrs.	Gangliosides	.....

* The number of min. designates the time required for solvent to travel 8 to 10 in. at 25 ± 1°C. (1) Acidic silicic acid paper, ascending technique, chamber lined with paper saturated with solvent just prior to insertion of papers. (2) 3 MM paper impregnated with 0.5 M dipotassium hydrogen phosphate, ascending technique, unlined chamber. (3) 3 MM paper impregnated with 1 M sodium carbonate solution, ascending, unlined chamber. (4) Neutral silicic acid paper, ascending chromatography, unlined chamber. (5) Whatman No. 4 paper, ascending chromatography, unlined chamber. All papers 7 in. x 9 in. (except for system 17 where papers 12 in. to 18 in. long are used) and development in chambers 10¾ in. x 2¾ in. x 10½ in. (l x w x h, internal dimensions) except for system 17 where a chamber 18 in. high and 12 in. in diameter is used.

the system is reduced by heating (100–120°C.) and the papers are kept in a dry atmosphere prior to chromatography, the capacity of the stationary phase to bind lipid is greatly reduced. The importance of water is further indicated by the fact that the order of migration of the lipid classes is not always altered by changing the nature of the salt or the organic substance in the stationary phase. When ion exchange is involved as well as hydrogen bonding, relative migrations may change.

The relative migration of phosphatidyl ethanolamine and phosphatidyl serine can be used to illustrate the role of ion exchange in the paper chromatographic process. Phosphatidyl serine has a carboxyl group in addition to the amino and phosphate groups present in phosphatidyl ethanolamine. When these two lipids are chromatographed in an acidic system, that is, where the stationary phase is sufficiently acidic (as with acid washed silicic acid) to depress the dissociation of the carboxyl group of phosphatidyl serine, the two lipids are not separated (7). When phosphatidyl serine in the salt form is chromatographed in a neutral or basic system, phosphatidyl serine is readily separated completely from phosphatidyl ethanolamine (7). The acidic stationary phase converts the carboxylate ion to the undissociated carboxyl group and this form of phosphatidyl serine is not separated completely from phosphatidyl ethanolamine. The demonstration of this difference by paper chromatography facilitated the development of the first column chromatographic procedure for the separation of phosphatidyl ethanolamine and phosphatidyl serine (6).

A very important form of interaction in paper chromatography of lipids was first appreciated in work with acidic lipids (6,7). Strong hydrogen bonds appear to be formed between anionic groups of a lipid (such as the carboxyl group of phosphatidyl serine) and anionic groups of an adsorbent (such as silicate) through water molecules. Awareness of the importance of this type of bonding occurred while studying the column chromatography of ionic lipids on the sodium and potassium forms of phosphocellulose. We had thought that the negatively charged phosphatidyl serine molecule might pass through the column ahead of phosphatidyl ethanolamine. We found however that phosphatidyl serine was more firmly retained by the phosphocellulose. This apparently paradoxical situation whereby a negatively charged substance is bound to a negatively charged stationary phase became understandable when it was realized that the sodium form of phosphocellulose failed to retain lipids at all in the absence of water. This work has been extended to other polar stationary phases which showed that similar behavior is exhibited by silicates, sulfates, and other anionic substances. This will be considered further in an accompanying report on column chromatography. The great strength of this type of binding probably arises from the greater negative charge of the carboxyl group of the anion.

In summary, it appears that the polar stationary phases used in the chromatography of lipids interact in three major ways: the dipole-dipole type of interaction is encountered with relatively nonpolar lipids (such as unsaturated hydrocarbons), while hydrogen bonding and ion exchange are important for the polar and ionic lipids.

Silicic acid is one of the most useful substances for impregnation of paper. Since Dieckert and Reiser (8), and Lea, Rhodes, and Stoll (9) first reported the successful use of silicic acid impregnated filter paper

for separations in the lipid field, other useful methods have been introduced from several laboratories (5,6,10,11). In the discussions below, the results of extensive investigations in this laboratory will be presented for a variety of lipids.

2. The preparation of silicic acid impregnated paper.

Silicic acid impregnated paper is prepared by dipping filter paper into a sodium silicate solution and then into mineral acid. The paper is then washed with water to remove excess acid and salt. Papers of widely different character that give different separations may be prepared using the same general technique.

The sodium silicate solution may be obtained commercially (a frequently used product is that supplied by Mallinckrodt) or may be prepared from silicic acid).

The Sodium Silicate Solution. (1) To 1 l. of distilled water in a 4-l. beaker add 280 g. of sodium hydroxide pellets. Stir until dissolved and cool in a basin of water. Add 400 g. of silicic acid slowly with stirring and cool to room temperature (the sp. gr. should be about 40–42° Bé.). Dilute with 500 ml. of distilled water and mix thoroughly. (2) Mallinckrodt sodium silicate solution (40–42° Bé.) is diluted with an equal volume of distilled water. (3) Mallinckrodt sodium silicate (40–42° Bé.) is diluted with an equal volume of 6 *N* KOH, shaken vigorously to mix and allowed to stand overnight to settle. The upper layer is used for impregnation of papers.

Impregnation and Drying of Paper. Whatman No. 1 or 3 MM paper for chromatography is cut into 7 x 11-in. pieces with the long dimension being in the direction of the grain (the machine direction should be indicated on the box containing the paper). Unwashed paper may be used satisfactorily with silicate solutions 1 and 3 above, but paper prewashed by capillary descent with 2 *N* acetic acid is preferable with silicate solution 2.

An amount of sodium silicate solution just adequate to impregnate the number of papers to be prepared is poured into a large glass or porcelain tray, and the papers are immersed in the solution for at least 5 min. The papers are removed and excess sodium silicate is removed by passing the paper gently over a smooth edge of the tray or by hanging on a glass rod and pushing the excess off by passing two glass rods, one on either side, down the paper. (The paper should not be allowed to dry prior to inserting in the hydrochloric acid solution. It is not necessary to let the paper stand in air prior to transfer to acid.)

The papers are next immersed in 6 *N* HCl acid in a glass tray. For each ten papers (No. 3 MM), 1 l. of 6 *N* acid is used with silicate solutions 1 and 3 above, or 250 ml. of 6 *N* acid saturated with NaCl for solution 2 above. The papers should be transferred to the tray individually and not allowed to touch for a minute or so (uneven coating results from papers sticking together before the surface silicate is converted to silicic acid). The papers are kept in the acid for 10 to 30 min.

Excess acid and salt are removed by transferring the papers to a glass tray containing 2 l. of distilled water (used for only 10 papers). Several types of paper that give different separations are made by controlling the number of water washes. It was found that two washes with 2 l. of distilled water gives an "acid" paper. The pH of the wash water at equilibrium is about 2. Four 2-l. washes give a "neutral" paper and the pH of the final wash at equilibrium should be about 5. Uniform washing is achieved by gently rocking the trays for several minutes and preventing papers from falling on top of each other and fail to equilibrate with the wash water. Each paper can be rinsed with a stream of distilled water between washes to increase the efficiency of each wash. The procedure must be standardized in each laboratory and the exact number of washes required for a given type of paper will depend upon the exact technique used (extent of agitation, etc.).

The acidity of the paper may be related to the migration of a fatty acid (e.g. oleic acid) in system 12 of Table I. On very acid paper the fatty acid migrates to or very near the solvent front. On very neutral paper fatty acid migrates about with lecithin (see Fig. 18).

The paper can be dried at room temperature or in an oven. As the amount of water in the paper affects chromatographic migration, the drying should be carried out in a uniform manner. Acid paper may be dried in an oven at 60–100°C. but should not be heated after it is just dry because it will become brittle and then turn brown. Neutral paper can be activated (free water removed) by heating at 100–120°C. until dry and then for an additional 10 to 30 min. The papers are placed in a polyethylene bag while hot when very dry paper is desired. Plastic bags are ideal for maintaining papers at any water content desired and best results are obtained if two bags, one inside the other, are used. Moderately dry paper is used for most purposes, but very dry or moist papers are sometimes useful.

Uneven deposits of silicic acid on the paper can be smoothed out by rubbing with a piece of soft paper or cloth. This is usually desirable when chromatograms are to be photographed.

Comments on Differences in Papers. The papers described above (prepared with No. 3 MM) have a high capacity and should have about 4 g. of silicic acid per sheet (about 25% of the weight of each paper). Papers with less silicic acid can be obtained by dilution of the sodium silicate solution to the desired level. Although papers impregnated with small amounts of silicic acid allow solvent to travel very fast (up to 4 times as fast as heavily impregnated paper), such papers do not have the high capacity of the heavily coated papers. We prefer thicker 3 MM paper with a maximum of silicic acid as more lipid (up to several hundred micrograms) can be applied to a small spot. This greatly increases sensitivity for purity checks and for the recognition of minor components in mixtures. Relatively short chromatograms are required for good separations. Once spots are just separated, further solvent migration is of little significance. When compounds migrate too close together, it is usually best to change solvent systems rather than extend running time for increased resolution.

3. Spotting of paper chromatograms.

Ideally, solutions of the lipids or other substances to be chromatographed should be sufficiently concentrated so that a suitable amount of material is obtained with a single application. Several applications with complete drying in between each one can be used to provide enough material without sacrifice of spot shape, provided each successive application is centered exactly on the point of the previous application and

the spot diameter does not change appreciably during successive applications. A suitable concentration range for a mixture may be found by preparing the lipid in a concentration of 2–5 mg./ml. and spotting 10–40 μl. volumes of solution (to give amounts of lipid equivalent to 20–200 μg. or more in some cases) followed by development of a trial chromatogram. When most of the lipid fails to migrate or migrates to the solvent front, more of the mixture can be applied and components that migrate within the body of the chromatogram may be detected at very low levels with ease. The examination of a nearly pure substance for trace impurities or chromatography of a standard can be carried out with 10–100 μg. of lipid in a single application of 10–20 μl. solution.

An appropriate solvent must be used for application. Generally a solvent should be selected that will readily elute all of the lipids to be examined from silicic acid. A solvent that will push the lipids to or near the solvent front is usually chosen. This insures uniform spreading of the material throughout the spot area. If a solvent that does not elute the lipids readily from silicic acid is used for application, the solvent may spread, but the lipid components may remain in the center of the spot (the silicic acid will retain the lipid and much of the lipid may remain at the initial point of contact). Thus petroleum ether or hexane should not be used for the application of neutral lipids, while benzene is frequently useful and chloroform is generally suitable. If the material remains at the center of the spot and does not spread properly, the chromatogram may be overloaded and elongated spots or streaks may be obtained.

Chloroform/methanol, 2/1, (v/v) is an ideal spotting solvent for mixtures of phospholipids. Mixtures with less methanol can be used where most of the lipid migrates relatively far down the paper. Pure cerebrosides can be prepared in concentrated solutions for spotting to paper in chloroform/methanol, 4/1, containing 2.5% pyridine or 0.5% water. Water is usually best as a small amount of pyridine may remain on the paper and give rise to a purple spot on the final chromatogram (see Figures 13,14).

Under some conditions the sensitivity for the detection of impurities or minor components of a mixture can be increased by choosing just the proper solvent for application. If most of the material fails to migrate from the origin or migrates to the solvent front, the sensitivity for small amounts of impurities that migrate in the body of the chromatogram can be improved by choosing a solvent that will not spread the lipid over the complete area covered by the solvent (the smaller spot size of lipid increases the ease of detection). A small amount of a relatively slow moving component in the presence of large amounts of substances that migrate further down the paper can sometimes be determined more readily with a solvent that does not completely spread the slow moving component over the area of the spot, while faster moving components are spread out almost completely. Under these conditions the smaller spot size for the minor components gives increased sensitivity. In general, a smaller amount of lipid may be detected if the spot size is very small. Thus, less than 1 μg. of most of the lipids can readily be localized on chromatograms when applied in 2–4 μl. of solvent. Application of extremely small spots has disadvantages. It is more difficult to recognize minor components of mixtures and more difficult to demonstrate spots clearly in photographs.

Proper spot shape can usually be obtained in ascending chromatography when the origin is 1 to 1.5 in. above the solvent level. The placement of the origin for descending chromatography depends upon the exact equipment used.

The migration characteristics of substances on paper chromatograms is influenced by the water content of the silicic acid paper. Water is an essential component of most of the chromatographic systems as discussed above. The water content is controlled in part by a uniform drying procedure and a uniform method of storing the paper prior to chromatography, as described above. In a very humid room, or when a very dry paper is desired, application of the sample must be carried out in a dry atmosphere. It is not possible to prevent some rehydration of paper other than by working in a dry box, but it is possible to greatly reduce rehydration by placing the paper between glass plates covered with clean dry paper. In this case only the small area to be spotted is exposed. Of course, this must be done immediately after the paper is removed from the dry storage container (plastic bag). After spotting, the papers are placed in a polyethylene bag, transported to the chromatography chamber, and transferred to the chamber from the bag. In this way it is possible to greatly reduce the water content of the system for comparison with results obtained with paper hydrated to any desired level.

4. Chamber equilibrium conditions and development of paper chromatograms.

The results obtained with a given developing solvent may vary quite widely depending upon the size of the chamber, the type of solvent, the presence or absence of a paper liner, and the number of papers placed in the chamber. With the extremely volatile solvents used for the nonionic lipids (hexane, ether, etc.), it is always wise to use a paper liner in the chamber. The paper liner is saturated with solvent just before the chromatograms are inserted for development. Saturation of the liner decreases evaporation of solvent from the paper during development.

The amount of solvent that evaporates from the paper during development is important for two reasons. First, the development time is considerably reduced if the atmosphere inside the chamber is more nearly saturated with solvent and evaporation from the chromatogram is decreased. Second, the extent of migration is altered as a result of the different solvent proportions created by unequal evaporation of components of a mixture.

Development time is usually reduced in a small chamber. When the ratio of surface area of the paper to total volume of chromatographic chamber is large, evaporation from the paper and development time are decreased. Because some solvent does leave the paper and solvent may be transferred from the atmosphere to the paper, it is evident that the composition of the mobile and stationary phases may change during the chromatographic process. Some change is inevitable with a mixture of solvents when chromatography is begun with solvent free paper in the usual manner. The more polar component(s) of the solvent mixture will drop out preferentially into the polar stationary phase as the mixture passes down the paper. The mobile phase is gradually depleted of the more polar component(s). Furthermore, the most volatile com-

ponents will tend to leave the paper to a greater extent and there will be a relative increase in the less volatile components. These factors must be kept in mind if reproducible results are to be obtained.

It is wise to keep in mind that when a small chamber with a saturated paper liner is used, the development time may be very short and the ratio of solvent components for optimum resolution may be different from the ratio that is required to give similar migration characteristics over a longer period of time in a larger chamber (with or without a liner). If one desires therefore to speed up a system that was originally developed for an unlined, relatively large chamber, a smaller chamber with a saturated liner can be used, but it may be necessary to change the solvent proportions in order to obtain the proper migration of components down the paper.

A satisfactory chamber size for ascending chromatography is 10¾ in. x 2¾ in. x 10½ in. (l x w x h, internal dimensions). The chambers may be obtained from Brinkman Instruments Inc., 115 Cutter Mill Road, Great Neck, L.I., N.Y. Two chromatograms are developed at a time. When a larger number of chromatograms are to be developed, a wider chamber can be used, although several small ones may be better. The small chambers are lined with paper held in place by glass rods cut to fit tightly and placed across each end of the chamber. The chromatogram is attached with a clip to another glass rod running the full length of the chamber and cemented into place with epoxy resin or calcium silicate. The clip prevents the papers from moving during development. The chromatographic solvent is poured into the chamber to a level of about 1 in. (about 400 ml.) and the chamber liner is saturated just before the papers are inserted by tilting the chamber first to one side and then the other. The top is sealed with silicone stop cock grease after the papers are inserted, the chamber is leveled to insure a straight solvent front (the edge of the paper should be straight and the paper inserted straight up and down). The composition of a solvent mixture inside the chamber will change due to evaporation and solvent must be replaced regularly.

There is little reason for development time to be appreciably decreased when the stationary phase is spread over a glass plate (thin-layer chromatography). When similar solvents and the same type of chamber are used for both paper and thin-layer chromatography, the cross-fibers of the cellulose constitute only a small, variable additional resistance to solvent migration; differences in development time are small.

5. Staining and Photography.

There are several useful and moderately specific color reactions that can be used for the location of some lipids. The ninhydrin reaction as described from this laboratory (6) is very useful for phospholipids with free amino groups (phosphatidyl ethanolamine, phosphatidyl serine, the corresponding lpsophosphatides, and some unknown and uncharacterized lipids encountered in tissues). The ammonium molybdate reagent as described by Levene and Chargaff (12 and originally believed to be rather specific for choline containing lipids, is of some value in confirming the presence of lecithin, sphingomyelin, or lysolecithin on chromatograms, but it is not entirely specific as compounds containing free amino groups give a light test. The Schiff's reagent (p-rosanilin decolorized with sulfurous acid has frequently been recommended for the detection of aldehyde-containing lipids (the plasmalogen forms of phospholipids). As has been pointed out (6), such reagents may be rather unpredictable and nonspecific staining may take place so that it is necessary to include standards of known plasmalogen forms and substances that do not contain aldehydes so that the degree of nonspecificity of the test can be determined.

The most useful general reagent for location of lipids on paper chromatograms is a 0.001% solution of Rhodamine 6 G in 0.25 M dipotassium hydrogen phosphate (6). This is a sensitive general stain for all classes of lipids. It can be used successfully with hydrocarbons, polar nonionic lipids, phospholipids, sulfatides, and the water soluble gangliosides. There is no known lipid class that does not stain with this dye solution under appropriate conditions.

Staining with Rhodamine 6 G is accomplished as follows: a small amount of dye solution is placed in a glass or porcelain tray and the paper chromatograms are immersed in the dye solution (usually face down) for at least 20 sec. Some substances stain better if left in the dye solution for several minutes while a few stain better if papers are removed after 1–2 sec. The papers are then removed, rinsed in distilled water, and viewed wet under ultraviolet light. Both long and short wave ultraviolet sources are satisfactory. Some lipids give bright yellow spots when the papers are viewed wet. Other lipids may give purple, orange-purple, yellowish-purple, or yellowish-orange spots. This color differential may be of value in distinguishing compounds, but the sensitivity of various colors is different and bright yellow spots against a purple background are best for photography. Silicic acid impregnated paper prepared as described above generally produces a paper giving a purple background when stained. Generally the spots are brighter if the chromatograms are stained just after they are removed from the chamber. With some systems a little residual organic solvent in the paper may increase the brightness of the spots after staining. Papers developed with solvents containing acids or pyridine should be heated for a few minutes as acid may prevent staining and pyridine may give a very dark purple background.

Purple and orange spots seen on wet chromatograms may become yellow and yellow spots may increase in intensity when the papers are partially dried. Drying may be accomplished rapidly in an oven, but more uniform results may be obtained at room temperature. In either case the chromatograms are examined at intervals until the maximum color develops. Some compounds give exceptionally bright spots when the papers are completely dry. Occasionally the background may be somewhat yellow. This can be decreased or converted entirely to a purple background by immersing papers that have been stained and dried in 0.02% pyridine or 1 *N* potassium hydroxide solution. The latter solution greatly increases the color intensity with fatty acids and some other lipids. These procedures require practice, but are simple and reproducible after a few trials. All of these aids for producing intense spots should be tried and standardized in each particular laboratory with each technique for preparing paper. Variations may be encountered with different batches of unwashed filter paper. The lower limit of identification is ordinarily somewhat less than 1 μg.

Photographic reproduction of paper chromatograms may be accomplished using an ordinary camera, but this form of photography is inconvenient for routine work. We have adopted a simple, rapid procedure using the Polaroid Land camera (Model 110B), as suggested to us by Dr. Michael Schotz. The camera is equipped with a photocopy lens and an orange filter and loaded with Type 42 or 44 (200 or 400 speed) film. The camera is mounted on a standard Polaroid Land camera copy maker and focused by viewing a chromatogram or other suitable object through a plate provided for this purpose. Once the adjustment on the photocopier is made, the camera may be removed and replaced without refocusing. We have replaced the lamps supplied with the photocopier with two short-wave ultraviolet lamps (Mineralite Model SL 2537 obtainable from Ultra Violet Products, Inc., South Pasadena, Calif.) that are held in place by laboratory clamps mounted on a ring stand. Two lamps are required for uniform illumination. The camera is set at f 8 and exposure times of 2–50 sec. are required depending upon the color and intensity of the background and the spots. When the background is very light, short exposure times are required. When the background is a deep purple, exposure times up to 50 sec. may be necessary (with type 42 film). The usual 10-sec. development time for the print is satisfactory, although good results may be obtained if the print is allowed to develop for several minutes. The print is then removed from the camera and the picture examined. If the exposure has not been satisfactory another photograph may be taken immediately. Ordinary visible light illumination may be used with the same equipment to photograph chromatograms after reaction with ninhydrin, etc.

6. Solvent Systems.

Substances varying in polarity from saturated hydrocarbons to the water soluble lipids (gangliosides) may be examined using the chromatographic systems described in Table I. The photographs illustrate the paper chromatographic migration of a variety of substances in the various solvent systems and serve to define these systems. As the table and figures are more or less self explanatory, emphasis will be placed on general relationships and the limitations of the various chromatographic systems.

(a) *Spot Size and Shape.* The size and shape of a spot on the finished chromatogram may not correspond to the size and shape applied to the paper. When too much material is applied as a round spot, the material may streak or spread back due to overloading. Streak-back may be caused by hydrolysis, oxidation, etc., and these causes must be distinguished from overloading by varying the load.

A smaller spot than the one applied can be observed on the finished chromatogram if lipid does not spread as far as the solvent during application as discussed above. A round spot, a bar-shaped spot, or a line all of greater diameter than the initial diameter of the round spot at the origin may be obtained. This is due to the lateral spread of the substance during the first rush of solvent over the origin. The mobile phase at this time contains the maximum amount of the more polar component(s) of the system and the lipid is pushed against the solvent front and may spread out as a line or bar. During development the more polar component(s) are lost to the stationary phase and the bar or line may reform into a round spot of larger diameter than the spot applied, or it may remain as a bar or line when the amount of lipid is small. This behavior can be observed directly with colored substances. The same phenomenon can give rise in two dimensional chromatography to a round spot smaller than the area over which the substance was applied at the origin. In this case, a line or bar-shaped area produced in the first dimension is pushed into a small round spot in the second dimension.

(b) *Nonionic Lipids.* Systems 1–9 of Table I are used for paper chromatography of substances varying in polarity from hydrocarbons to monoglycerides and ceramides. Generally speaking these systems fractionate lipids into classes on the basis of the type, number, and position of polar functional groups without appreciable effects due to differences in unsaturation or length of the hydrocarbon chains. Maximum effects from differences in chain length and unsaturation are observed for system 1 (hexane as solvent). Saturated hydrocarbons are readily separated from the unsaturated hydrocarbon squalene (see Fig. 1), fatty aldehydes fractionate (Fig. 26), and both sterol esters and waxes (esters of fatty acids and fatty alcohols, Fig. 1) separate to a certain extent due to differences in chain length. Cholesterol palmitate and cholesterol acetate are completely separated as are methyl palmitate and myristyl palmitate.

Migration is affected to a great extent by water in the paper with system 1. With very dry papers squalene has an R_f value of about 0.5 and waxes and sterol esters migrate just off the origin. Paper containing more water is usually more useful as all the classes migrate well off the origin (Fig. 1).

Systems 2 and 3 give complete separations of sterol esters and waxes differing greatly in chain length, but with more polar substances and with all other systems even these differences are not observed.

Substitution of halogen for hydrogen does not alter migration. This was established with α-brompalmitic acid and β-cholesteryl chloride.

Ring compounds and aliphatic compounds with the same polar functional group(s) such as cholesterol and long chain aliphatic alcohols are completely seperated with several solvents. Closely related ring compounds differing in side chain length or unsaturation are not separated in the same systems (cholesterol, ergosterol, and β-sitosterol). The difference between some ring and aliphatic compounds is illustrated by separation of fatty acid esters of long chain aliphatic alcohols from the corresponding sterol esters (cholesterol palmitate and myristyl palmitate) (Fig. 1).

Different polar functional groups increase polarity to various extents. A carbonyl group increases polarity and fatty aldehydes are readily separated from hydrocarbons with system 1 (see Figs. 1 and 26). Similar results are obtained when cholestane-3-one (Fig. 2) and keto acids are compared to the parent compounds without keto groups. An ester carbonyl gives added polarity when compared to an ether linkage as illustrated by the migration of 1-monopalmitin and batyl alcohol (a 1-glyceryl ether, Figs. 11, 12). An ester group confers almost the same polarity as the carbonyl group of an aldehyde. This is well illustrated by the migration of fatty aldehydes, sterol esters, and waxes in system 1 (Figs. 1 and 26).

The most pronounced effects are produced by differences in number and position of hydroxyl groups.

Compounds with one hydroxyl group migrate in the body of the chromatogram in systems 2 to 5. These systems are useful for the separation of monohydroxy sterols, monohydroxy aliphatic alcohols, and diglycerides (Figs. 2–10). Systems 4 to 7 are useful for compounds with 2 hydroxyl groups (monoglycerides, α-glyceryl ethers, dihydroxy aliphatic alcohols, dihydroxy sterols, etc., Figs. 7–12), and systems 8 and 9 are used for compounds with 2 to 4 hydroxyl groups. Beef brain ceramide (fatty acid amide of sphingosine) has 2 hydroxyl groups when a nonhydroxyl fatty acid is linked to sphingosine (2 free hydroxyl groups) and 3 hydroxyl groups when an hydroxy fatty acid is linked to sphingosine. Similarly yeast cerebrin (also a ceramide) that differs from animal ceramide in that the yeast phyto-type sphingosine contains 3 hydroxyl groups may have 3 or 4 hydroxyl groups depending upon whether a nonhydroxy or hydroxy fatty acid is linked to sphingosine. Compounds with 5 or more hydroxyl groups are run in systems for phospholipids.

When determining the number of hydroxyl groups in a molecule to predict migration, an isolated carboxyl group can be counted as an hydroxyl group. Fatty acids migrate with fatty alcohols when the chromatogram is developed with ether-hexane mixtures (Figs. 5,6), while they are just separated with benzene as solvent (Fig. 3). Deoxycholic and cholic acids migrate in systems 8 and 9 as substances with 3 and 4 hydroxyl groups, respectively. Hydroxy acids show a somewhat different behavior. The 2-hydroxy acid is less polar than the 3-hydroxy, etc., so that position effects are strong and it is not until the carboxyl group is well separated from the hydroxyl group (isolated) that the maximum polarity is reached (Figs. 7–9). The equivalence of the carboxyl group and the hydroxyl group can be considered as a position effect. Thus, while fatty acids, aliphatic alcohols, and the methyl esters of 2-hydroxy fatty acids migrate to the same position with ether-hexane mixtures as developing solvents, the methyl esters of 3-, 4-, 5-, etc. hydroxy acids where the carbonyl and hydroxyl groups are separated do not migrate as far and are separated from monohydroxy alcohols, fatty acids, and 2-hydroxy fatty acid methyl esters.

The position of the polar groups in compounds with two or more polar groups exerts a marked effect upon migration. A complete series of synthetic hydroxypalmitic acids (2–16) was available for examination, as well as the corresponding methyl esters and keto palmitic acids. These three series of compounds show clearly the effect of position. When the hydroxyl (or carbonyl) group is moved away from the carboxyl or ester group, the polarity of the molecule increases and migration of the substance is decreased (see Figs. 7–9). The change is not uniform as indicated in the various photographs. Migration decreases steadily from 2 to the 6 position for the methyl esters of the hydroxypalmitic acids and then increases and decreases again. The hydroxypalmitic acids migrate in a somewhat different manner from the corresponding methyl esters so that it is possible in most cases to place the position of the hydroxyl group precisely on the hydrocarbon chain by examining both the fatty acid and its methyl ester. Chain length and unsaturation do not influence migration of the hydroxy fatty acids or their methyl esters as shown by correspondence in migration of the 2-hydroxypalmitic, stearic, and hexacosanoic acids, and ricinoleic, 12-hydroxystearic, and 12-hydroxypalmitic acids, as well as for 10- and 12-hydroxystearic and palmitic acids. As chain length and unsaturation do not influence migration of these lipid classes, the synthetic hydroxypalmitic acids can be used as standards on chromatograms in the study of hydroxy fatty acids from natural sources as described below (see Figs. 22–24 in particular).

Other illustrations of position effects are the separation of 1- and 2-monoglycerides and the 1,2- and 1,3-diglycerides (Fig. 3). The effect of methyl groups adjacent to hydroxyl groups is illustrated by the relative migration of cholesterol and lanosterol (Fig. 2). Methyl groups on position 4 in lanosterol appear to decrease the effect of the hydroxyl group on position 3 and lanosterol and cholesterol are completely separated.

Certain special features of some of the solvents should be pointed out. System 3 (5% ether in hexane) is very useful for the separation of several important biological substances (Figs. 4 to 6, 23, 25, 27). The K vitamins, the tocopherols (vitamins E), coenzyme Q, and vitamins A and D_2 are all separable. A positive identification of each class can be made as both vitamin K_1 and K_2, α-, β-, and γ-tocopherols, and the coenzyme Q's of different chain lengths migrate together or so close to each other that there is no reason for confusion.

Benzene is of considerable value as a solvent (system 2) despite the fact that development time is somewhat longer than with ether-hexane mixtures. While fatty acids and long chain monohydroxy aliphatic alcohols migrate to the same position in the ether-hexane systems, they are separable using benzene as solvent. Development time with system 2 can be decreased by elevating temperature. This is possible as there is no selective evaporation with a single solvent. Another feature of this single component system is that the chamber may be deoxygenated by passing pure nitrogen through the system prior to chromatography without altering solvent proportions. This can be accomplished when the chromatographic chamber is fitted with appropriate inlet and outlet holes. The chromatogram is suspended by a small magnet from the top of the chamber, the chamber flushed with nitrogen, and the magnet outside the chamber removed to allow the chromatogram to drop into the developing solvent. Labile substances may be examined by this method.

Whatman No. 3 MM paper impregnated by treating with a 2 M sodium carbonate solution can be substituted for silicic acid paper and chromatograms can be developed in about 20 min. with 5% ether in hexane. Results are similar to those obtained with silicic acid paper (system 3), but the sensitivity is not as great with the carbonate paper.

(c) *Highly polar and ionic lipids (cerebrosides, phospholipids, sulfatides, gangliosides).* The highly polar cerebrosides and the ionic lipids require more polar solvents for chromatography. Figures 13 to 21 illustrate the general chromatographic behavior of many of the compounds commonly encountered and show how small amounts of impurities can be detected.

Cerebroside from beef brain gives two spots in some systems. With systems 14 and 15, two widely separated spots are obtained (Figs. 13,14). This is clearly the separation of two different groups of cerebrosides,

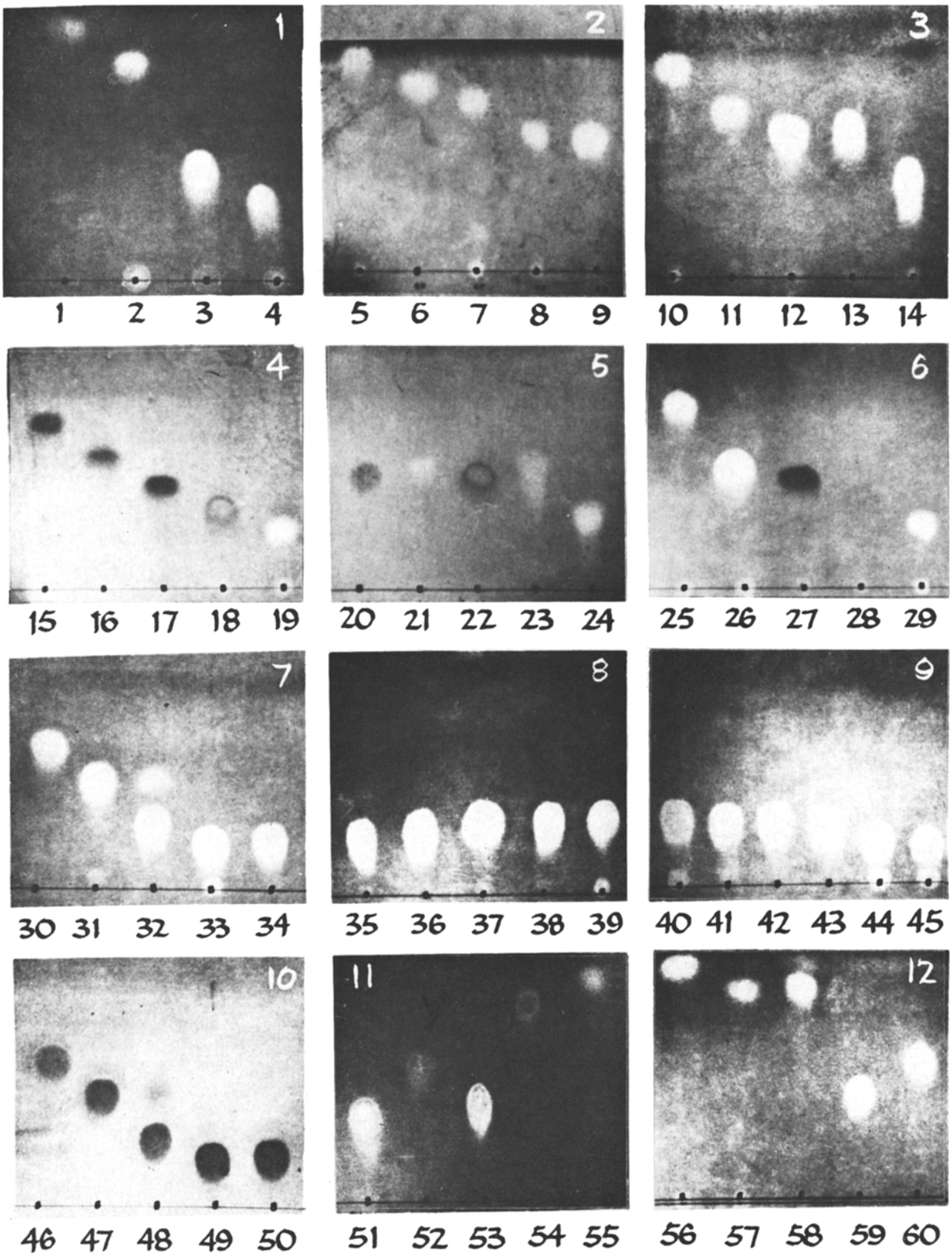


PLATE I

(All chromatograms reduced to approximately ⅓ actual size)

the slower moving component containing hydroxy fatty acids and the faster moving containing nonhydroxy fatty acids.

A more complex situation is encountered with sulfatides (the sulfate esters of cerebrosides). Sulfatides tend to separate into four spots and spots tend to be slightly diffuse. With systems 13 and 16 the spots are well defined and separated when a very small amount of material is applied. Part of the separation is related to the presence of two groups of sulfatides, those containing hydroxy fatty acids and those containing nonhydroxy fatty acids (as with cerebrosides). This can account for two spots. Further separation can be accounted for by the presence of another group of sulfatides with an additional free hydroxyl group on sphingosine or by a difference in the position of the free hydroxyl group of sphingosine. As beef brain ceramide and yeast cerebrin (also a ceramide) show multiple spotting not accountable for by ionic differences or total number of hydroxyl groups, position effects are suggested. Position effects could be related to the presence of erythro and threo forms and their partial separation, or to the presence of the secondary hydroxyl group of sphingosine on a carbon other than carbon three. Position effects from the hydroxyl group of fatty acids have been excluded as only 2-hydroxy fatty acids are present (see description below and Figs. 22,24,29). Position effects related to the hydroxyl groups of sphingosine are indicated by several findings. First, the phenomenon is seen only with sphingosine and sphingolipids. Second, it is seen with both ionic and nonionic sphingolipids. Third, the effect with sulfatide is abolished with pyridine in the developing solvent (diisobutylketone/pyridine/water, 55/45/5). Only one spot is obtained from sulfatide in this case as there is no separation of sulfatides with nonhydroxy and hydroxy fatty acids. Evidently the weaker position effects related to hydroxyl group placement should not influence migration in this case. The separation into more than two spots is not seen with cerebroside or sphingomyelin. The fact that yeast cerebrin, a ceramide with three free hydroxyl groups on the phytosphingosine portion of the molecule, gives more than four spots in system 8 suggests that epimeric substances may be involved. Sphingosine hydrochloride obtained by hydrolysis of beef brain cerebroside and sulfatide with hydrochloric acid separates into four spots with system 16. It is thus possible that different forms of the erythro and threo modifications are involved.

System 15 gives the largest number of individual spots with complex lipid mixtures of the various systems for polar lipids. Sterols, sterol esters, glycerides, fatty acids, ceramides, and glyceroldiacylphosphatidic acids migrate to the solvent front. These compounds migrate as a thin line and do not stain brightly because the dye does not penetrate the compact area. Varying the proportions of the mixture, as with systems 14 and 16, decreases or increases polarity and migration. Some ceramides migrate back from the solvent front with system 14 and sulfatides and sphingosine migrate well away from the origin in system 16. System 9 is useful for ceramides (particularly yeast cerebrin). System 12 gives a different order of migration from system 15 and is useful for confirmation of the identifications made with other systems. Systems 10 and 11 with either phosphate or carbonate paper give different orders of migration from systems 12 to 16 and should be used for confirmation of identifications. Orders of migration are different with phosphate and carbonate papers as well. When all of the systems are used, a fairly reliable tentative identification can be made or a compound can be shown to be different from known standards.

System 13 is useful for sulfatides because the four spots are separated in a strongly acidic system. Papers impregnated with dipotassium hydrogen phosphate or sodium carbonate (systems 10 and 11) are of particular value when complex lipid mixtures are to be examined for phosphatidyl ethanolamine, lysophosphatidyl ethanolamine, and acidic lipids. Since neutral lipids (sterols, sterol esters, and glycerides) as

Legend for Plate I

FIG. 1. System 1, moderately dry paper, spots 1–4, 20 μg. each of (1) n-dotriacontane, (2) squalene, (3) myristyl palmitate, and (4) cholesterol palmitate.

FIG. 2. System 2, spots 5–9, 20 μg. each of (5) beef spleen triglyceride (isolated from a magnesium silicate column), (6) cholestane-3-one, (7) lanosterol, (8) cholesterol, and (9) dihydrocholesterol.

FIG. 3. System 2, spots 10–14, 20 μg. each of (10) beef spleen triglyceride, (11) palmityl alcohol, (12) cholesterol, (13) oleic acid, and (14) a mixture of 1,2- and 1,3-diglycerides (separated into 2 spots).

FIG. 4. System 3, spots 15–19, 10 μg. each of (15) vitamin K_2, (16) α-tocopherol, and (17) coenzyme Q_{10} (gift from Dr. Karl Folkers), (18) 5 μg. oleic acid, and (19) 20 μg. cholesterol. Photographed at a time when oleic acid gave a purple-orange spot. (Compare with fatty acid spots, Figures 6 to 10).

FIG. 5. System 3, spots 20–24, 10 μg. each of (20) oleyl alcohol, (21) stearyl alcohol, (22) myristyl alcohol, (23) 1-hydroxyoctatriacontane, and (24) cholesterol.

FIG. 6. System 3, spots 25–29, 20 μg. each of (25) cholesterol palmitate and (26) beef spleen triglyceride, (27) 10 μg. coenzyme Q, (28) 5 μg. oleic acid, and (29) 10 μg. cholesterol. Photographed at a stage where the light purple spot of fatty acid was barely visible. (Compare with fatty acid spots, Figures 4, 7 to 10.)

FIG. 7. System 4, spots 30–34, 20 μg. each of synthetic 2-,3-, 4-,5-, and 6-hydroxymethylpalmitates, respectively. Note the presence of a very small amount of the 3 isomer in the 4-hydroxy preparation, and very small amounts of the free fatty acids in the 3- and 5-hydroxy preparations. Note in particular the complete separation of 2-hydroxymethylpalmitate (spot 30) from the other isomers and compare the migrations of the 2 to 6 isomers with the 7 to 16 isomers in Figures 8 and 9.

FIG. 8. System 4, spots 35–39, 20 μg. each of synthetic 6-,7-, 8-,9-, and 10-hydroxymethylpalmitates, respectively. (Compare with other isomers, Figures 7 and 9.)

FIG. 9. System 4, spots 40–45, 20 μg. each of synthetic 11-,12-,13-,14-,15-, and 16-hydroxymethylpalmitates, respectively. Compare with other isomers (Figures 7 and 8).

FIG. 10. System 4, spots 46–50. Same chromatogram shown in Figure 7 photographed when completely dry to illustrate the color change from yellow to purple when the wet chromatogram is dried. (Compare with fatty acid spots, Figures 4 and 6.)

FIG. 11. System 6, spots 51–55, (51) 20 μg. of 1,5-hexadecandiol, (52) 5 μg. batyl alcohol, (53) 20 μg. of 1-monopalmitin, and 5 μg. each of (54) 2-hydroxypalmitic acid, and (55) palmitic acid.

FIG. 12. System 6, spots 56–60, 20 μg. each of (56) beef spleen triglyceride, (57) cholesterol, (58) diglyceride, (59) 1-monopalmitin, and (60) batyl alcohol.

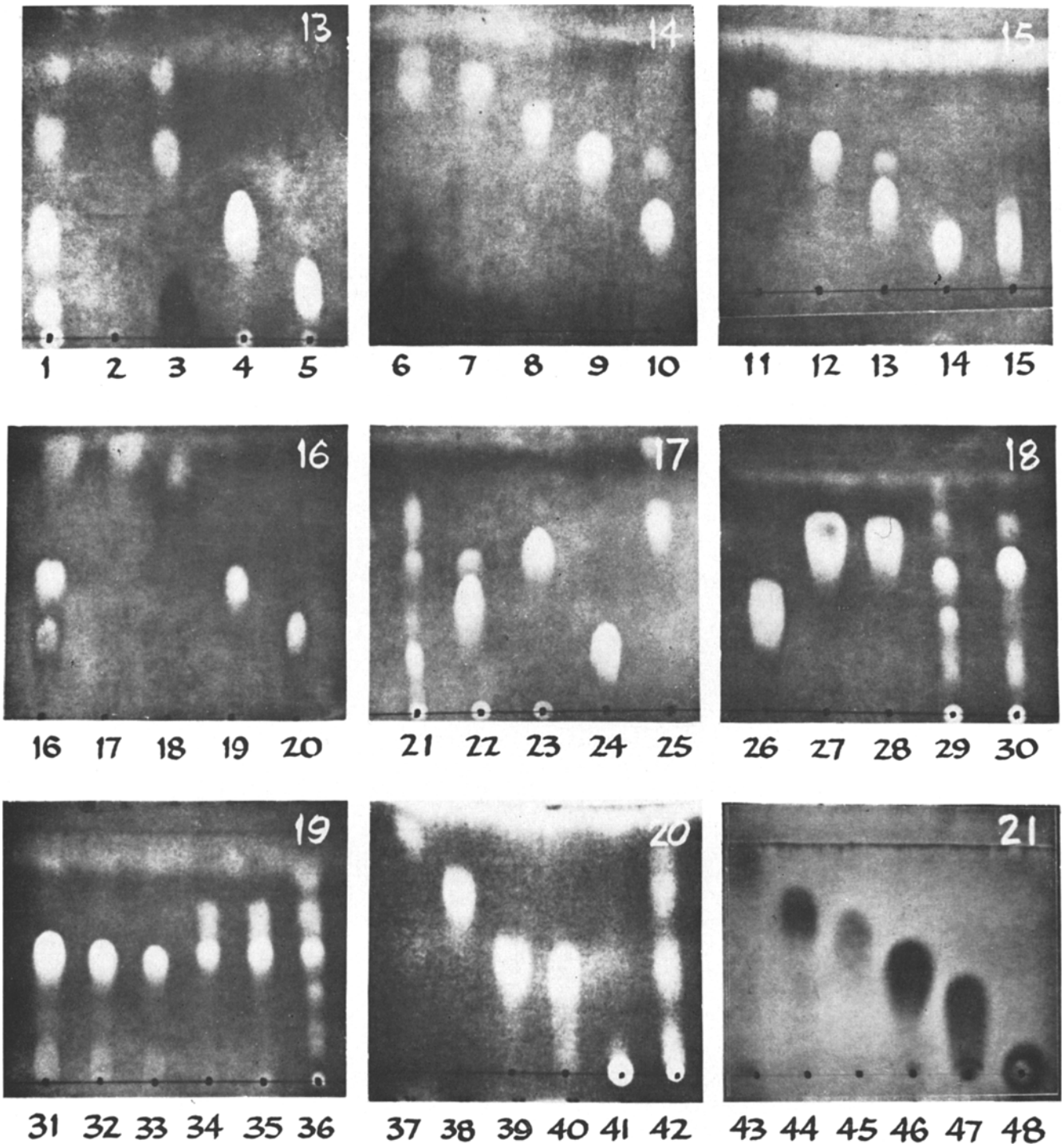


PLATE II

(All chromatograms reduced to approximately ⅓ actual size)

well as two of the major phospholipids, lecithin and sphingomyelin, migrate to the solvent front on phosphate paper, the examination of a complex mixture for very small amounts of acidic lipids or phosphatidyl ethanolamine is accomplished with ease and high sensitivity. These systems are useful also for the recognition of free fatty acids in complex lipid mixtures. Nonhydroxy fatty acids migrate just behind phosphatidyl ethanolamine and hydroxy fatty acids migrate just behind and overlap slightly on short runs with the nonhydroxy fatty acids (Fig. 21).

The relative migrations for the commonly encountered highly polar lipids (cerebroside, sulfatide, ganglioside, lecithin, sphingomyelin, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, lysophosphatidyl ethanolamine, lysolecithin, glyceroldiacylphosphatidic acid, and cardiolipin, Figs. 13 to 21) are different enough so that any one of them can

be distinguished from the others when examined in several systems. It may not always be possible, however, to distinguish each of these lipids in a complex mixture. This limitation arises from the fact that different lipids migrate to or near the same position in different systems. Although one dimensional paper chromatography alone is not always suitable for the recognition of all of the constituents of a complex mixture, it is a powerful tool when coupled with column chromatography. Column chromatography on silicic acid or any other single adsorbent is limited in the same way.

(d) *Paper chromatography in the study of hydrolysis products of lipids: Products from whole beef brain lipid, cerebrosides, sulfatides, phosphatidyl ethanolamine, and phosphatidyl serine.* Paper chromatography can be used as a simple and conclusive method for the study of hydrolysis products of complex lipids. The finding that 2-hydroxy fatty acids and their methyl esters can be distinguished readily from the isomeric compounds with hydroxyl groups on other carbon atoms made possible the examination of animal brain cerebrosides and sulfatides for isomeric hydroxy fatty acids. These two lipids contain large

Legend for Plate II

FIG. 13. System 15, spots 1–5. Dry (heat activated) paper was used and spotting was done with paper between glass plates to prevent rehydration. (1) Mixture of 30 μg. of cholesterol, ceramide, cerebroside, lecithin, and sphingomyelin isolated from beef brain as the front fraction from a DEAE cellulose column (eluted with chloroform/methanol, 7/1, v/v). Spots 2–5, fractions obtained from the mixture shown in spot 1 after elution from a silicic acid-silicate-water column. (2) 30 μg. of cholesterol that stains lightly as it moves as a small line at the solvent front. (3) 30 μg. of cerebroside fraction. Upper white spot cerebroside with nonhydroxy fatty acids, and lower white spot cerebroside with hydroxy fatty acids. The dark spot just off the origin was formed from pyridine. Chloroform/methanol, 4/1, containing 2.5% pyridine to solubilize cerebroside was used for spotting in this case only. (4) 30 μg. lecithin fraction, and (5) 30 μg. sphingomyelin fraction (both fractions pure, as shown by this chromatogram). Compare with Figure 16 showing migration of the same fractions in system 12.

FIG. 14. System 15, spots 6–10. The paper was not heat activated and spotting was not carried out with paper under glass plates. On "moist" paper all compounds migrate farther down the paper but relative migrations are unchanged. (Compare with Figure 13 on dry paper.) (6) 20 μg. cerebroside (the two cerebroside spots and the dark spot from pyridine as in Figure 13); (7) 20 μg. beef brain phosphatidyl ethanolamine (isolated from a DEAE cellulose column); (8) 20 μg. beef brain phosphatidyl serine (isolated by solvent partition and chromatography on magnesium silicate); (9) 20 μg. lecithin isolated from soybean (the commercial soybean phospholipid mixture, Asolectin, in chloroform/methanol, 5/1, was treated with Norit activated charcoal until all other lipids were adsorbed); (10) 20 μg. beef heart sphingomyelin prepared by the alkaline degradation procedure of Rapport and Lerner (J. Biol. Chem., *232*, 63, 1958). Note the presence of a small amount of residual lecithin in the preparation.

FIG. 15. System 12, with moderately dry paper (heat activated but not spotted between plates). Spots 11–15, 20 μg. each of (11) phosphatidyl ethanolamine, (12) lecithin, (13) sphingomyelin containing a small amount of lecithin, (14) lysolecithin, and (15) phosphatidyl serine. Lysolecithin was produced from soybean lecithin by snake venom degradation in ether (7). The lower lysolecithin phase was washed with ether to remove free fatty acids, dried, and passed through a DEAE cellulose column to free it of traces of enzyme. The other preparations were the same as for Fig. 14. The small spot ahead of the major spot in the phosphatidyl serine preparation is the plasmalogen form (strongly positive to the Schiff's reagent) just separated from the large amount of diester form.

FIG. 16. System 12, spots 16–20 same as spots 1–5 of Figure 13. Dry paper (heat activated and spotted between glass plates). Note that cholesterol migrates to the solvent front and cerebroside gives one spot just behind the solvent front (both compounds usually stain lightly after development in this system).

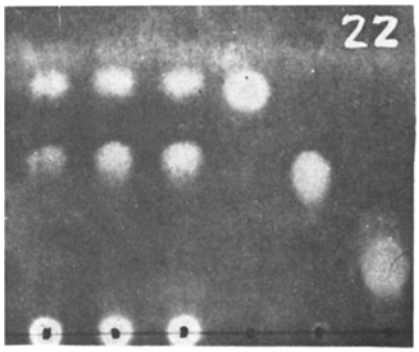
FIG. 17. System 12, spots 21–25. Moderately dry paper (heat activated but not spotted between plates) showing increased migration of all compounds, compared to Figure 16 with dry paper. (21) 30 μg. egg yolk "lecithin" fraction prepared by ethanol precipitation and alumina chromatography. From above down, phosphatidyl ethanolamine, lecithin, lysophosphatidyl ethanolamine, and lysolecithin followed by two slower moving uncharacterized components, (22) 30 μg. sphingomyelin preparation as in Figure 14. (Note the presence of residual lecithin.) (23) 30 μg. of soybean lecithin preparation as in Figure 14. (24) 30 μg. of lysolecithin as in Figure 15. (25) 30 μg. of a glyceroldiacylphosphatidic acid preparation contaminated with neutral lipid (migrating to the solvent front). Note that phosphatidic acid migrates with phosphatidyl ethanolamine.

FIG. 18. System 12. To illustrate the method of testing the acidity of silicic acid impregnated paper (see text for discussion) and results with complex lipid mixtures. The paper in this case was moist (not heat activated) and "neutral" as judged by the relative migration of hydroxy fatty acids with sphingomyelin and nonhydroxy fatty acids between phosphatidyl ethanolamine and lecithin. On more acidic paper fatty acids migrate with or ahead of phosphatidyl ethanolamine depending upon the degree of acidity. (26) 20 μg. of 2-hydroxypalmitic acid; (27) and (28) 40 and 20 μg., respectively, of palmitic acid; (29) 80 μg. of total beef brain lipid. The individual components are, from above down, cholesterol plus cerebroside, phosphatidyl ethanolamine, lecithin (cerebroside sulfate overlaps with lecithin), sphingomyelin, phosphatidyl serine, inositol phosphatide, and ganglioside at the origin. (30) 80 μg. of commercial soybean phospholipid mixture. The spots, from above down, are phosphatidyl ethanolamine, lecithin, unknown material migrating in the position of sphingomyelin, phosphatidyl inositol, and uncharacterized material at the origin. Note that soybean lecithin migrates slightly ahead of beef brain lecithin. Soybean lecithin also migrates slightly ahead of egg yolk lecithin (Figure 17). The soybean lecithin contains primarily unsaturated fatty acids in both the 1 and 2 positions, while the brain and egg yolk lecithins contain unsaturated fatty acids primarily in the 2 position.

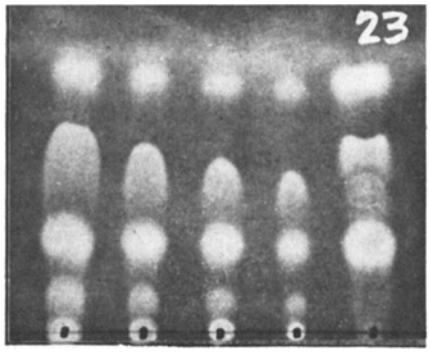
FIG. 19. System 12. The chromatogram was stained and partially dried before it was photographed. Spots 31–33 from 60, 40, and 20 μg., respectively, of a commercial synthetic preparation of dimyristyl lecithin. The major component is lecithin, but impurities are evident. Spots (34) and (35) from 100 and 50 μg., respectively, of total lipid from beef heart mitochondria (prepared by Dr. Sidney Fleischer, Enzyme Institute, Madison, Wisconsin). Individual components, from above down, are phosphatidyl ethanolamine, cardiolipin, lecithin, and barely detectable spots in the sphingomyelin and inositol phosphatide areas of the chromatogram. Spot 36 is from 80 μg. of total beef brain lipid. Individual components as in Figure 18. Note that mitochondrial and brain lecithins migrate to the same position, but that the synthetic lecithin (only saturated fatty acids) does not migrate quite as far as the unsaturated lecithins. See also Figure 18 where soybean lecithin is seen to migrate ahead of beef brain lecithin.

FIG. 20. System 10 with 3 MM paper impregnated with 1 M sodium carbonate solution. Spots 37–41 from 20 μg. each of (37) lecithin, (38) sphingomyelin, (39) lysolecithin, (40) phosphatidyl ethanolamine, and (41) phosphatidyl serine, and (42) total beef brain lipid. Samples prepared from sources and by methods indicated for Figure 15. Compare with Figure 21 and note the light streak back of phosphatidyl ethanolamine (spot 40) indicating autoxidation products.

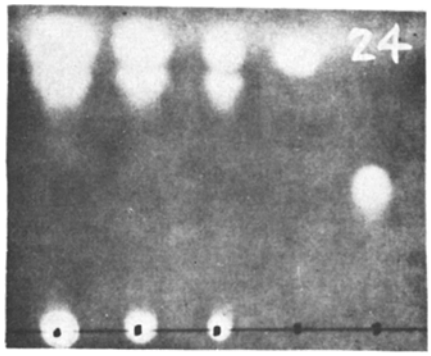
FIG. 21. System 10 with 3 MM paper impregnated with 0.5 M dipotassium hydrogen phosphate. Chromatogram stained, dried, dipped in 1 N potassium hydroxide and dried again to show dark spots and color differences. Spots 43–48, 30 μg. each of (43) lecithin, (44) lysolecithin, (45) phosphatidyl ethanolamine, (46) palmitic acid, (47) 2-hydroxypalmitic acid, and (48) phosphatidyl serine. (Compare with Figure 20.)



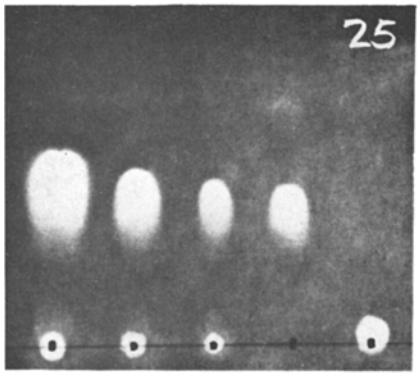
1 2 3 4 5 6



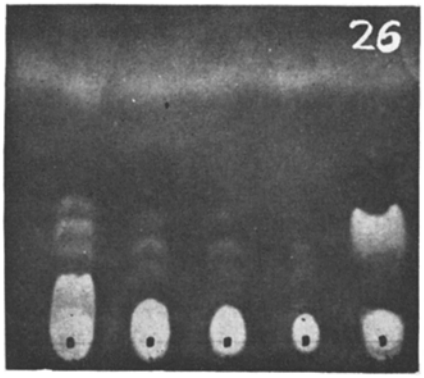
7 8 9 10 11



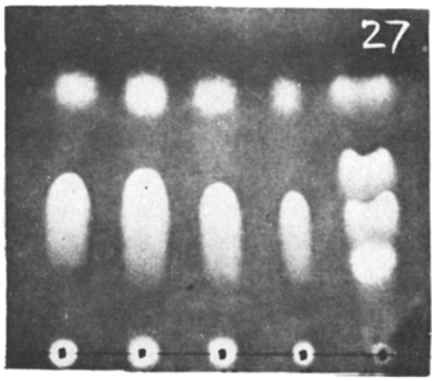
12 13 14 15 16



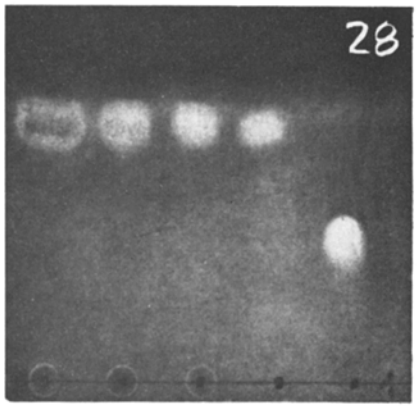
17 18 19 20 21



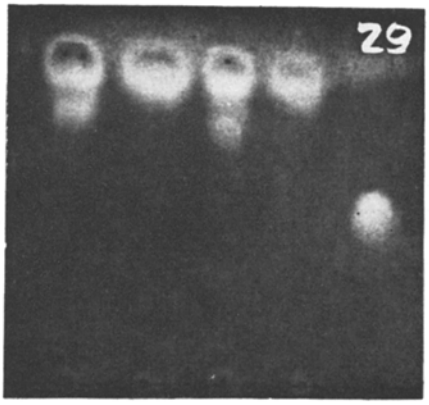
22 23 24 25 26



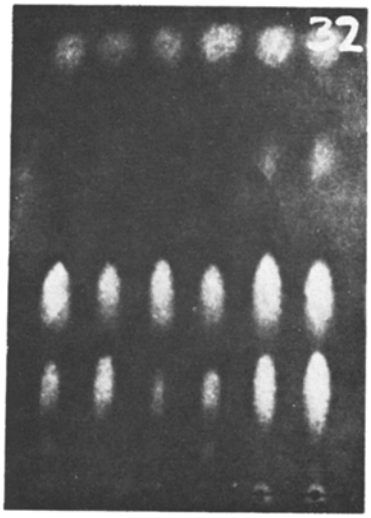
27 28 29 30 31



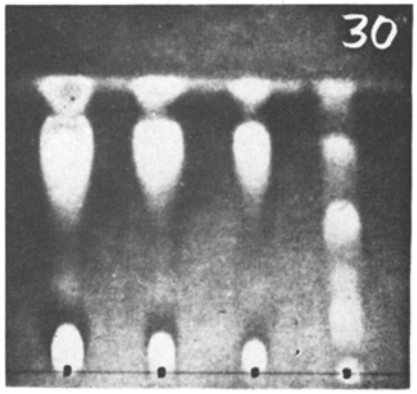
32 33 34 35 36



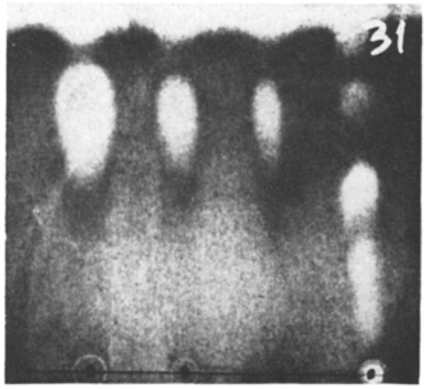
37 38 39 40 41



50 51 52 53 54 55



42 43 44 45



46 47 48 49

PLATE III

(All chromatograms reduced to approximately ⅓ actual size)

amounts of hydroxy fatty acids and only 2-hydroxy fatty acids have been isolated, although other isomers have not been excluded.

For the study of possible position isomers of hydroxy fatty acids, beef brain cerebroside was isolated in quantitative yield by elution from magnesium silicate and diethylaminoethyl cellulose (as described in an accompanying report) and a mixture of cerebroside and sulfatide was isolated from magnesium silicate from both mouse and rabbit brains. The preparations were free of other lipids (demonstrated by paper chromatography and confirmed by infrared spectroscopy) and were representative of the lipids as they occur in brain as quantitative recovery was obtained. Twenty mg. of each of the preparations was hydrolyzed in 3 ml. of 3 *N* hydrochloric acid in a sealed tube at 100°C. for 1.5 hrs. These conditions degrade cerebroside and sulfatide completely to release all fatty acid and sphingosine. The contents of the tubes were extracted into n-hexane, the solutions

Legend for Plate III

FIG. 22. System 5, spots 1–6. Fatty acids (hexane soluble material) from acid hydrolysates of brain cerebroside and cerebroside plus sulfatide (see text for details) to show presence of 2-hydroxy fatty acids only (no isomers). (1) 30 μg. lipid from mouse brain cerebroside plus sulfatide. The fast moving component in each case is fatty acid, the middle spot 2-hydroxy fatty acids, and the material at the origin is sphingosine hydrochloride. (4) 20 μg. palmitic acid, (4) 20 μg. 2-hydroxypalmitic acid, and (6) 20 μg. 6-hydroxypalmitic acid. (Compare with Figures 7, 8, and 9 where relative migrations of methylhydroxypalmitate isomers are shown.)

FIG. 23. System 3. Hexane soluble lipid from acid hydrolysate of total beef brain lipid (see text for details). Spots 7–10 correspond to 400, 200, 100, and 50 μg. respectively, of lipid hydrolysate. Individual components from above down, in each case, are fatty aldehydes, fatty acids, cholesterol, 2-hydroxy fatty acids, and sphingosine hydrochloride, (11) a mixture of 20 μg. each of, from above down, cholesterol palmitate, beef spleen triglyceride, oleic acid, and cholesterol.

FIG. 24. System 6. The chromatogram was stained, dried, dipped in 1 N potassium hydroxide and photographed while wet. Hexane soluble lipid from 48-hr. acid hydrolysate of total beef brain lipid (see text for details) to show absence of batyl alcohol or related α-glyceryl ethers in the hydrolysate. Spots 12–14 from 400, 200, and 100 μg., respectively, of lipid. From above down, in each case the components are fatty aldehydes plus fatty acids, 2-hydroxy fatty acids, and sphingosine hydrochloride at the origin. (15) 20 μg. palmitic acid and (16) 20 μg. batyl alcohol. Note the complete absence of material in the batyl alcohol region demonstrating the absence of ether linkages in glycerophosphatides of beef brain.

FIG. 24. System 3. Chromatogram stained, dried, dipped in 1 N potassium hydroxide and photographed wet. Hexane soluble lipid from 1 hr. acid hydrolysate of beef brain phosphatidyl serine (see text for details). Spots 17–19 correspond to 120, 60, and 30 μg., respectibely, of beef brain phosphatidyl serine. (20) 20 μg. palmitic acid, and (21) 20 μg. batyl alcohol. The major spot in the hydrolysates is from fatty acids. The small amount of material at the origin is from a partial hydrolysis product (probably a lysophosphatidic acid) that disappears when the hydrolysis time is increased to 3 hrs. Note absence of a fatty aldehyde spot (a trace could be seen at the solvent front but faded before photography). Hydroxy fatty acids and α-glyceryl ethers are absent from the hydrolysate. Note the high capacity of system 3 for fatty acids. The spots enlarged in the horizontal direction during development (see text for full discussion of factors affecting spot size and shape).

FIG. 26. System 1. Chromatogram-stained and partially dried before photography. Hexane soluble lipid from 1-hr. acid hydrolysate of total beef brain lipid (see text for details). Spots 22–25 correspond to 400, 200, 100, and 50 μg., respectively, of lipid. The small light staining spots are fatty aldehydes (Schiff's positive). The other material at the origin and just off the origin is mainly fatty acid. (26) Control mixture of 20 μg. each of (from above down) cholesterol palmitate, beef spleen triglyceride, and cholesterol (the latter two substances did not migrate from the origin).

FIG. 27. System 3. Chromatogram stained and partially dried before photography. Hexane soluble lipid from 1-hr. hydrolysate of beef brain phosphatidyl ethanolamine (see text for details). Spots 27–30 correspond to 100, 200, 100, and 50 μg., respectively, of lipid. From above down, components are fatty aldehydes, fatty acids, and a small amount of a partial hydrolysis product (probably a lysophosphatidic acid) this disappears after hydrolysis for 3 hrs. (31) A control mixture of 20 μg. each of (from above down) cholesterol palmitate, beef spleen triglyceride, oleic acid, and cholesterol.

FIG. 28. System 6. Hexane soluble lipid from 3-hr. acid hydrolysate of beef brain phosphatidyl ethanolamine (see text for details). Spots 32–34 correspond to 200, 100, and 50 μg., respectively, of lipid. The single spot in each case is from a mixture of fatty aldehydes and fatty acids. (35) 20 μg. palmitic acid, and (36) 20 μg. batyl alcohol. Note absence of material at the origin demonstrating the absence of partial hydrolysis products, and the absence of material in the batyl alcohol region that demonstrates the absence of glyceryl ethers and hence of ether linkages in beef brain phosphatidyl ethanolamine.

FIG. 29. System 6. Hexane soluble lipid from 48-hr. acid hydrolysates of total beef brain lipid and beef brain phosphatidyl ethanolamine to show the absence of partial hydrolysis products and α-glyceryl ethers in the hydrolysates. Spots (37) and (39) are from 400 and 200 μg., respectively, of total brain lipid hydrolysates, and spots (38) and (40) from 400 and 200 μg., respectively, of phosphatidyl ethanolamine hydrolysate. Spots from above down are fatty acid plus fatty aldehydyes, and hydroxy fatty acids. (41) 20 μg. batyl alcohol).

FIG. 30. System 12. Chromatogram stained, dried, dipped in 1N potassium hydroxide and photographed wet. Hexane soluble lipid from 1-hr. acid hydrolysate of beef brain phosphatidyl ethanolamine to show the presence of small amounts of highly polar partial hydrolysis products (contrast with Figure 31). Spots 42–44 correspond to 80, 40, and 20 μg., respectively, of lipid from the phosphatidyl ethanolamine hydrolysate. From above down, the components are fatty aldehydes, fatty acids, and three different partial hydrolysis products. The bright spot just off the origin is thought to be a lysophosphatidic acid. (45) 80 μg. total beef brain lipid (see Figure 18 for list of individual components). Compare with Figure 31 where partial hydrolysis products are shown to be absent after 3 hours.

FIG. 31. System 12, spots 46–49. The same as spots 42–45 of Figure 30 but from a 3-hr hydrolysate of phosphatidyl ethanolamine. No partial hydrolysis products are visible. This, plus the absence of α-glyceryl ethers in such hydrolysates (Figure 28), demonstrates the absence of ether linkages in beef brain phosphatidyl ethanolamine.

FIG. 32. System 12. Dry (heat activated) paper and spotted with paper between glass plates. Chromatogram stained and partially dried before photography. Total lipids extracted from blood of normal adult and child with Niemann-Pick disease (see text for discussion). Spots 50 and 51, 100 μg. total lipid from whole blood of patient and normal control, respectively; spots 52 and 53, 100 μg. of total lipid from blood plasma of patient and normal; spots 54 and 55, 100 μg. total lipid from erythrocytes of patient and normal. Individual components are, from above down, a mixture of neutral lipids (at solvent front and including sterols, sterol esters, free fatty acids, glycerides), phosphatidyl ethanolamine (visible in erythrocyte samples), lecithin, sphingomyelin, and lysolecithin (seen primarily in whole blood and plasma). All samples from the patient show a decrease in sphingomyelin compared to the control. This is illustrated clearly with plasma lipids where the patient level (spot 52) is about 50% of the control level (spot 53). Lecithin in all samples from the patient appears to be elevated.

evaporated to dryness, lipid dissolved in chloroform, the solutions spotted on acid silicic acid paper, and chromatograms developed with solvent 5 (with which the various isomeric hydroxy fatty acids can be distinguished). As shown in Fig. 12, only 2-hydroxy fatty acids were obtained from the three sphingolipid preparations. The absence of other isomers is clear and the sensitivity of the method is great. The presence of fatty aldehydes, fatty acids, and 2-hydroxy fatty acids only in crude beef brain lipid hydrolyzed in sealed tubes in 2 *N* hydrochloric acid for 1 hr, and for 48 hrs. was clearly demonstrated by chromatography with systems 4 and 6, as shown in Figs. 23, 24, and 29.

Paper chromatography is of great value in the study of hydrolysis products of the glycerophosphatides. This is illustrated by the ease with which beef brain phosphatidyl ethanolamine and phosphatidyl serine hydrolysates can be examined for the presence of fatty aldehydes, fatty acids, and α-glyceryl ethers. The findings with phosphatidyl ethanolamine will be described to illustrate the method of examination. Pure phosphatidyl ethanolamine was isolated from beef brain by elution from DEAE cellulose in the acetate form with 30% methanol in chloroform as described in an accompanying report. The lipid was isolated in quantitative yield, and paper chromatography and infrared spectroscopy were used to demonstrate the absence of other lipids. The preparation had very low molar extinction coefficients at 235 and 275 mμ (317 and 165, respectively), indicative of the absence of autoxidation (6). Twenty mg. of lipid was hydrolyzed in 3 ml. of 2 *N* HCl in sealed tubes for 1, 3, and 6 hrs. at 100°C. and for 24 and 48 hrs. at 110°C., the lipid was washed from the tubes with n-hexane, the hexane was removed by evaporation under nitrogen, the lipid was dissolved in chloroform, spotted on acid silicic acid paper, and examined in systems 1, 3, 6, 12, and 15. The lipid was white after 1 hr., showed a trace of color at 3 and 6 hrs., and finally became light yellow in color with a small amount of black sediment at 24 and 48 hrs. System 1 demonstrated the presence of several fatty aldehydes in the hydrolysate. System 3 showed fatty aldehydes at the solvent front, fatty acids in the middle of the chromatogram, and material at the origin that did not migrate (Fig. 27). System 6 showed the complete absence of any lipid in the batyl alcohol region in all hydrolysates (Figs. 24,28,29). The presence of a lysophosphatidic acid-like substance in the 1 hr. hydrolysate was demonstrated with systems 12 (Fig. 30) and 15. This substance was not found after 3 hrs. (Fig. 31).

When complete hydrolysis of phosphatidyl ethanolamine to fatty acids and fatty aldehydes was demonstrated at 3 hrs. (no partial hydrolysis products were observed), there was not trace of α-glyceryl ether in the hydrolysates (Figs. 28,29). Control hydrolystates with batyl alcohol and methyl linolenate under the various conditions used for the lipid samples demonstrated the stability of both batyl alcohol and fatty acid to the hydrolysis conditions. The findings may be taken as relatively clear demonstration of the absence of ether linkages in beef brain phosphatidyl ethanolamine. Beef brain phosphatidyl serine and total beef brain lipid were hydrolyzed and examined in the same way. No α-glyceryl ether was found in either of the preparations (Figs. 24 and 29 illustrate the findings with total brain lipid). After only 1 hr. at 100°C. (2 *N* HCl), phosphatidyl serine was almost completely degraded to free fatty acid (Fig. 25) with only a trace of a partial hydrolysis product, and a small amount of fatty aldehyde. Complete hydrolysis of phosphatidyl serine is obtained after 3 hrs. under the same conditions.

(e) *Paper Chromatograph as a Survey Method.* Paper chromatography is widely used as a means for determining the nature and number of constituents in column chromatographic fractions. This use has been illustrated before (6,7). The method is valuable also as a survey technique prior to more extensive column chromatographic studies.

Fig. 32 illustrates the use of paper chromatography in a preliminary survey of erythrocyte lipids of patients with lipid storage diseases. Patients with inherited metabolic diseases may be expected to show within the erythrocytes, at least in some cases, the accumulation of the lipid stored in other tissues. As the erythrocyte can be sampled readily during life at frequent intervals, it was of particular interest to examine cells from various patients in order to determine whether or not column chromatographic fractionations would be profitable.

It was readily determined that the erythrocytes contained ganglioside by paper chromatography with system 17 followed by staining with Rhodamine 6 G. The ganglioside spot on paper chromatograms was distinctly larger in erythrocyte samples from patients with Tay-Sachs disease, but was of normal intensity in patients with Niemann-Pick disease, Gaucher's disease, and an uncharacterized type of demyelinating disorder. These findings are in keeping with the general conclusion that gangliosides accumulate (particularly in brain) in Tay-Sachs disease.

The phospholipid composition of the plasma and red cells in both Niemann-Pick and Tay-Sachs disease appeared to be different from the control samples. In both cases sphingomyelin was decreased (Fig. 32). It was surprising that sphingomyelin should be decreased in the erythrocytes of patients with Niemann-Pick disease as this substance has been described as the major lipid accumulated in other organs of the body. It is thus a distinct possibility that erythrocytes express the inherited metabolic disorder in a different manner.

Such provocative survey studies by paper chromatography are of limited value until the tentative identifications and semiquantitative estimations made by comparisons with graded amounts of authentic compounds are confirmed by careful quantitative isolations by column chromatography with proof of the purity and structure of the lipids by other methods. Such data are useful however as they indicate the methods that should be employed for the column chromatographic fractionation of the particular lipid mixtures and the compounds of special interest. The lipid distribution of erythrocytes from normal children and children with Tay-Sachs, Niemann-Pick, and Gaucher's disease at different ages can be studied rapidly by paper chromatography. When the reproducibility of lipid composition and major variables have been explored by paper chromatography, quantitative isolation of each of the lipid classes and analysis of the fatty acid composition of each lipid class can be undertaken by column chromatography with a great deal more assurance that the results are representative.

Pitfalls in Paper Chromatography

There are many ways in which erroneous conclusions can be drawn from paper chromatograms. The two spots from substances such as cerebrosides must not be confused with other lipids. Conversely, it must not be concluded that a single spot on a chromatogram is always produced from a single substance. Examples of different classes of lipids that migrate together are given above. One dimensional paper chromatography with a single solvent system is of very limited value and, even when several different systems are used, completely reliable identifications cannot be made. Paper chromatographic results must be confirmed by isolation, infrared examination, hydrolysis, etc.

Improper use of color tests for location of lipid spots can give rise to confusion. Even major lipid components can be missed if staining is not carried out under suitable conditions, particularly when a complex lipid is examined for the first time.

A common source of difficulty is autoxidation. More than one spot or streaking may be observed when readily oxidizable substances, such as phosphatidyl ethanolamine, are examined. Proper precautions must be taken to prevent autoxidation. The results with autoxidized phosphatidyl ethanolamine and phosphatidyl serine have been described in some detail (6). Other lipids including lecithin, if allowed to stand under unfavorable conditions for a long time, may show streaking and/or extra spots from autoxidation. The oxidation artifacts usually take the form of streaks backward from the major spot with a definite tendency to form relatively discrete spots in some cases. The more discrete spots indicate that some of the oxidation products are moderately stable. Some oxidation products fail to migrate from the origin.

Material remaining at the origin on silicic acid impregnated paper should not always be judged to be the result of autoxidation. Column chromatographic data indicates that small amounts of divalent ions present in silicic acid may cause retention of phospholipids, particularly lecithin and sphingomyelin. The influence of divalent ions in silicic acid impregnated paper is further indicated by the appearance of light streaks back from the major spot (observed particularly with lecithin and sphingomyelin).

Another important point to be appreciated when judging purity, particularly of phospholipids, is the fact that appreciable amounts of extraneous materials that do not stain at all or stain lightly may be present. We have encountered several substances of this type. Inorganic substances will not stain and gangliosides (water soluble lipids) give a light purple color with Rhodamine 6 G on phosphate paper and can be missed in low concentrations. Gangliosides can be detected however in small amounts after chromatography in system 17. In this case, ganglioside stains readily with Rhodamine 6 G to give bright yellow spots against a purple background (water soluble nonlipids do not stain and do not interfere with the detection of ganglioside). Inorganic substances can be detected and identified by infrared spectroscopy.

Several other artifacts may be introduced by the use of a strongly acidic silicic acid paper or by the use of strongly acidic solvents for paper chromatography. The application of the labile plasmalogen forms of the phospholipids (particularly of phosphatidyl ethanolamine) to acidic silicic acid paper may result in an appreciable hydrolysis of the α,β-unsaturated ether bond in the plasmalogen to give a lysophosphatide. This behavior is particularly pronounced with pure phosphatidyl ethanolamine. When a lipid mixture appears to contain lysophosphatides, hydrolysis during application to the paper must be excluded. Some oxidation products of phosphatidyl ethanolamine migrate on paper chromatograms like lysophosphatidyl ethanolamine and this again can give rise to the erroneous conclusion that lysophosphatide is present in a mixture.

Acid hydrolysis and autoxidation can give rise to erroneous interpretations when two-dimensional paper chromatography is used. Labile lipids can be applied to neutral silicic acid paper without hydrolysis and chromatograms can be developed with mixtures containing acetic acid without hydrolysis of plasmalogens during chromatography. The absence of streaking, trailback, or decrease of spot size and intensity on long runs proves that no appreciable hydrolysis has taken place during chromatography. When the finished paper chromatogram is dried in air, the more volatile components of solvent mixtures (as in systems 14 to 16) leave the paper. Acetic acid and water remain and some hydrolysis can take place in aqueous acetic acid during drying. Furthermore, some oxidation is inevitable during air drying, particularly with phosphatidyl ethanolamine. Two-dimensional chromatography with the same solvent in both directions may disclose more than one spot from phosphatidyl ethanolamine in the second dimension from hydrolysis and/or oxidation. The development of a paper chromatogram in two directions with the same solvent is a good test of the stability of compounds during drying of the paper chromatogram. The additional spots seen on such chromatograms must not be interpreted as new substances.

Acknowledgments

Special thanks are due to Richard Ray whose assistance in the preparation of the photographic illustrations was invaluable and to Robert J. Meyer for the samples of 2 to 16 hydroxypalmitic acids. The experimental work was supported in part by Grants C-3134 and B-1847 from the U. S. Public Health Service and Research Grant No. DA-CML-18-108-61-G-7 from the U. S. Army Chemical Research and Development Laboratories, Army Chemical Center, Maryland. Support of one of us (N. N.) by the Medical Research Foundation of Oregon is gratefully acknowledged.

REFERENCES

1. Kaufmann, H.P., and Nitsch, W.H., Fette, Seifen, Anstrichmittel, *56*, 154–158 (1954).
2. Mangold, H.K., Lamp, B.G., and Schlenk, H., J. Am. Chem. Soc., *77*, 6070–6072 (1955).
3. Kaufmann, H.P., and Schnurbusch, H., Fette, Seifen, Anstrichmittel, *61*, 523–528 (1959).
4. Kaufmann, H.P., and Kirschnek, H., Fette, Seifen, Anstrichmittel, *61*, 750–759 (1959).
5. Rouser, George, Marinetti, G.V., Witter, R.F., Berry, J.F., and Stotz, E., J. Biol. Chem., *223*, 485–497 (1956).
6. Rouser, George, O'Brien, J., and Heller, D., J. Am. Oil Chemists' Soc., *38*, 14–19 (1961).
7. Rouser, George, Bauman, A.J., and Kritchevsky, G., Am. J. Clin. Nutrition, *9*, 112–123 (1961).
8. Dieckert, J.W., and Reiser, Raymond, J. Am. Oil Chemists' Soc., *33*, 535–537 (1956).
9. Lea, C.H., Rhodes, D.N., and Stoll, R.D., Biochem. J., *60*, 353–363 (1955).
10. Marinetti, G.V., Erbland, J., Kochen, J., Federation Proc., *16*, 837–844 (1957).
11. Rouser, George, White, S.G., and Schloredt, D., Biochim. et Biophys. Acta, *28*, 71–80 (1958).
12. Levine, C., and Chargaff, E., J. Biol. Chem., *192*, 465–479 (1951).