THE CHROMATOGRAPHY OF PHOSPHATIDES ON SILICIC ACID-IMPREGNATED FILTER PAPER*

M. H. HACK

Departments of Medicine and Anatomy, Tulane University School of Medicine, New Orleans, La. (U.S.A.)

with the technical assistance of

CHARLOTTE W. LEATHERMAN

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The techniques of paper chromatography have enjoyed wide usage in biochemical research and can be expected to facilitate the solution of many difficult analytical problems. This report is concerned with our analysis of some of the more pertinent variables evident in the chromatography of phosphatides on silicic acid-impregnated paper. Our understanding of the limitations of various solvent combinations and of the characteristics of a variety of silicic acid-impregnated cellulose filter papers has provided us with a versatile system of analysis which we have extensively employed on a number of problems to be reported elsewhere.

The desirability of utilizing several solvent systems has already been discussed[1]; in the present communication we will describe a neutral system (2,6-dimethyl-4-heptanone–methanol–water), an acidic system (2,6-dimethyl-4-heptanone–acetic acid–water), and a basic system (2,6-dimethyl-4-heptanone–pyridine–water) and will demonstrate the necessity of employing all three solvent systems for the thorough analysis of the phosphatides.

MATERIALS AND METHODS

1. *Chromatography jars*

Three sizes of chromatography jars were used:

(a) 35 × 200 mm (Kimble No. 20065) contained 15 ml of solvent mixture. Short runs were made (1–2½ h) using 30 × 140 mm paper strips cut at the bottom as shown in Fig. 1a to prevent their clinging to the vessel wall. Trimming in this way resulted in more uniform movement of the solvent. These vessels were capped by polyethylene stoppers, through which a stainless steel wire projected to serve as a hanger for the paper. The principal use of these small jars was to determine the optimal ratio of solvent components necessary for the desired chromatographic separation (*cf.* Figs. 2–5) and were therefore used in batteries of 4–7 as necessary.

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(b) 150 × 300 mm (Corning No. 6942) contained 200 ml of solvent and employed 140 × 140 mm paper; these jars, capped by a glass plate containing a center hole for the hanger, were used for our normal analytical run of 2–6 h (*cf.* Fig. 1b, right-hand jar).

(c) 150 × 450 mm (Corning No. 6942) contained 200 ml of solvent and employed 140 × 290 mm paper; these jars were used when runs longer than 6 h were necessary for greater separation of the various phosphatides (*cf.* Fig. 1b, left-hand jar).

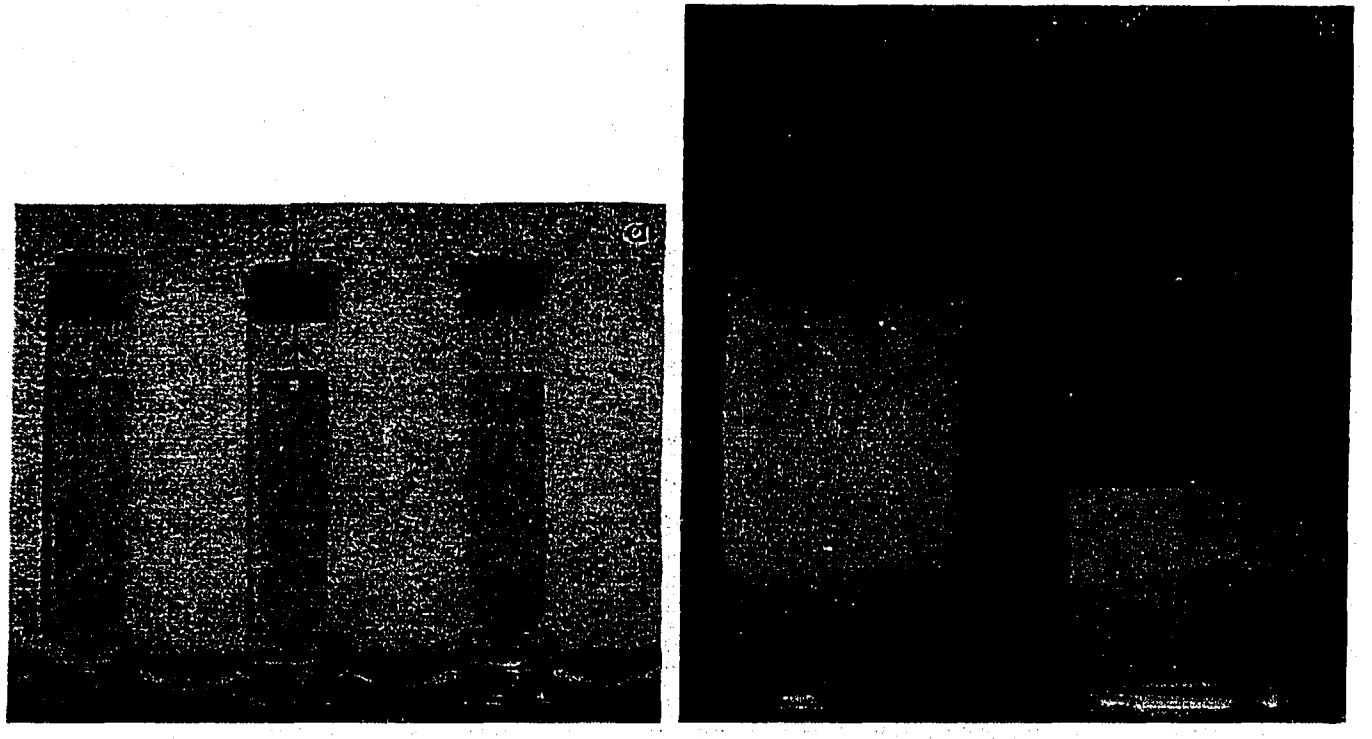

Fig. 1. (a) Chromatogramming in the small jars; normally only one sample was placed on each paper; however, with care to keep the area of sample application small, 2–3 extracts could be quite satisfactorily examined on each paper. (b) 6–8 samples were chromatogrammed on the wide papers shown here; our experience has indicated that an intermediate length paper would probably be the most generally useful.

2. Impregnation of paper

Potassium silicate was freshly prepared by slowly (during 5 min) pouring a slurry of 200 g silicic acid (100 mesh powder, Mallinckrodt or Baker Analytical reagent) in 400 ml water into a freshly prepared solution of potassium hydroxide (113 g Baker Analyzed Reagent in 400 ml H_2O) using a 2 l beaker and stirring briskly with a teflon-covered magnetic stirrer. This solution was cooled to room temperature, diluted to 1 l, and filtered through pyrex wool into a 220 × 340 mm pyrex tray. The filter paper (140 × 290 mm) was immersed in this solution for 5 min, let drain vertically for 5 min, then immersed in 6 *N* HCl for 10 min. The HCl was removed by running tap water 30 min and 3 ten-minute changes of distilled water. In this way excessive washing, which tended to leach the silicic acid from the paper, reducing its capacity, was avoided. After drying at room temperature, they were extracted by immersion

in chloroform–methanol (2:1, v/v) and again air dried. Papers impregnated by various dilutions of this 200 g/l solution were also studied; their lower capacity was generally of no advantage. Equivalent impregnations were obtained from a 5:2 dilution of DuPont's Potassium Silicate Solution (Electronics No. 200 Technical).

Clear solutions of a number of commercial sodium silicate preparations (40–42° Be) diluted 1:1, in general, made papers of low capacity, resulting in streaking and with less uniform properties than the two above; these papers tended to tear due to an excess of alkali, making handling difficult and were often full of bubbles due to an excessive carbonate content of the silicate solutions.

The general features to be described were observed with all of the following filter papers impregnated as described, although only Whatman No. 1 and Schleicher & Schüll 2043b were extensively examined: Whatman Nos. 1, 2, 3MM, 4, 5, 7, 11, 20, 31 Double, 40, 41, 41H, 42, 50, 52, 54; Schleicher & Schüll Nos. 2043b, 598. There is no doubt that a careful screening could lead to the selection of one or more of these papers especially useful for specific analyses. As a rule, the thicker papers had greater capacity due to their heavier impregnation.

3. *Solvents*

Our interests in the acid-labile plasmalogens[1,2] stimulated a search for non-acidic solvent systems of equal usefulness in the chromatography of these phosphatides. The simple substitution of pyridine or methanol for the acetic acid in the 2,6-dimethyl-4-heptanone mixtures of MARINETTI AND STOTZ[3] proved to be encouraging. The optimal ratios of the various solvents was ultimately determined by trial resulting in the following three solvent systems: (a) 2,6-dimethyl-4-heptanone–methanol–water 100:25:4; (b) 2,6-dimethyl-4-heptanone–acetic acid–water 100:40:8; (c) 2,6-dimethyl-4-heptanone–pyridine–water 100:75:10. It was found desirable to adjust the ratio of water for each batch of paper to achieve the desired separations (*cf.* Figs. 2–4). This was simply determined in a single run by using the small chromatography jars, the first containing no water, the second 1 part of water, the third two parts, etc., making use of as many jars as necessary. In a similar way the optimal ratio of heptanone:methanol, heptanone:acetic acid, and heptanone:pyridine was determined (*cf.* Fig. 5). The 2,6-dimethyl-4-heptanone (Matheson, technical grade) was purified by passing it through a 30 × 340 mm column containing 25 g Mallinckrodt Silicic Acid 100 mesh, overlaid by 50 g Woelm neutral Aluminium Oxide, activity grade 1; this column effectively removed a yellow contaminant from 500 ml of solvent. Alternately 500 ml of heptanone was shaken with 50 g Al_2O_3 and 2 g Norite and filtered. All other solvents were of analytical reagent grade and were used without further purification. The methanol and the pyridine containing systems were employed at room temperature, while the acetic acid system was used in a cold room at 2° in order to reduce hydrolysis of plasmalogens, although the short runs (1–2 h) at room temperature appeared to be safe and to give better resolution than similar runs in the cold (*cf.* Fig. 4). All three systems were found to be quite stable over many days, provided they were protected from evaporation, and required no equilibration. Chloroform–

Fig. 2. One hour small jar run at room temperature to demonstrate the influence of H_2O concentration in effecting resolution of infarct plasmalogen 8 and cardiolipin 7. Extract was from 24 h infarcted dog heart[1] chromatogrammed on Whatman No. 1 silicic acid-impregnated paper in ketone 100, methanol 25, and water from left to right 0, 1, 2, 4, 6 and 8. Plasmalogen and rhodamin stain.

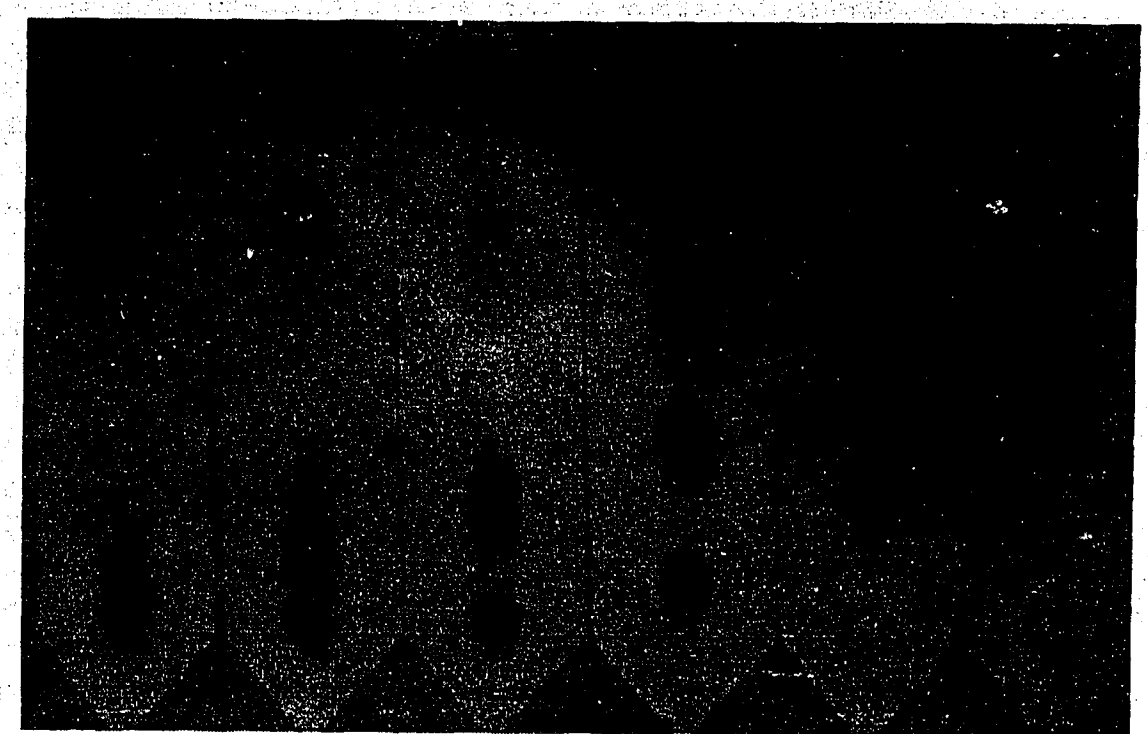

Fig. 3. One hour room temperature run of normal monkey heart extract. Whatman No. 1 impregnated paper in ketone 100, pyridine 75, and water left to right 0, 1, 2, 4, 6 and 8. With some papers 8 parts of water was sufficient to reduce the mobility of cardiolipin to that of phosphatidyl ethanolamine.

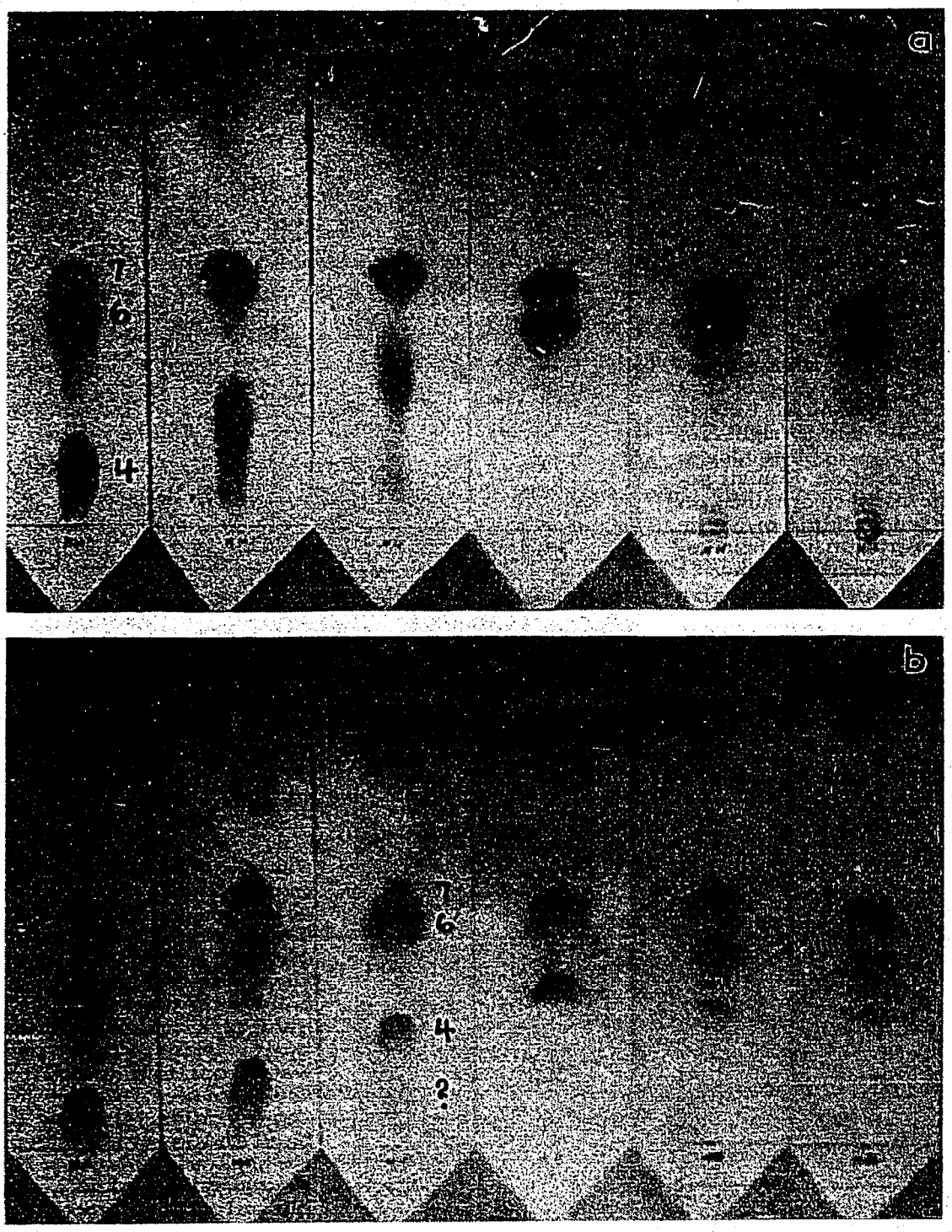

Fig. 4. Same extract and paper as Fig. 3, in ketone 100, acetic acid 40, water from left to right 0, 1, 2, 4, 6 and 8; (a) 3 h at 2°. (b) 1 h at room temperature, plasmalogen and rhodamin stain. The material labeled ? had properties resembling lysolecithin in this solvent system, but not in either of the other solvent systems as was demonstrated by chromatogramming this extract with lysolecithin. Notice that cardiolipin 7 was resolvable only in mixtures of low water content.

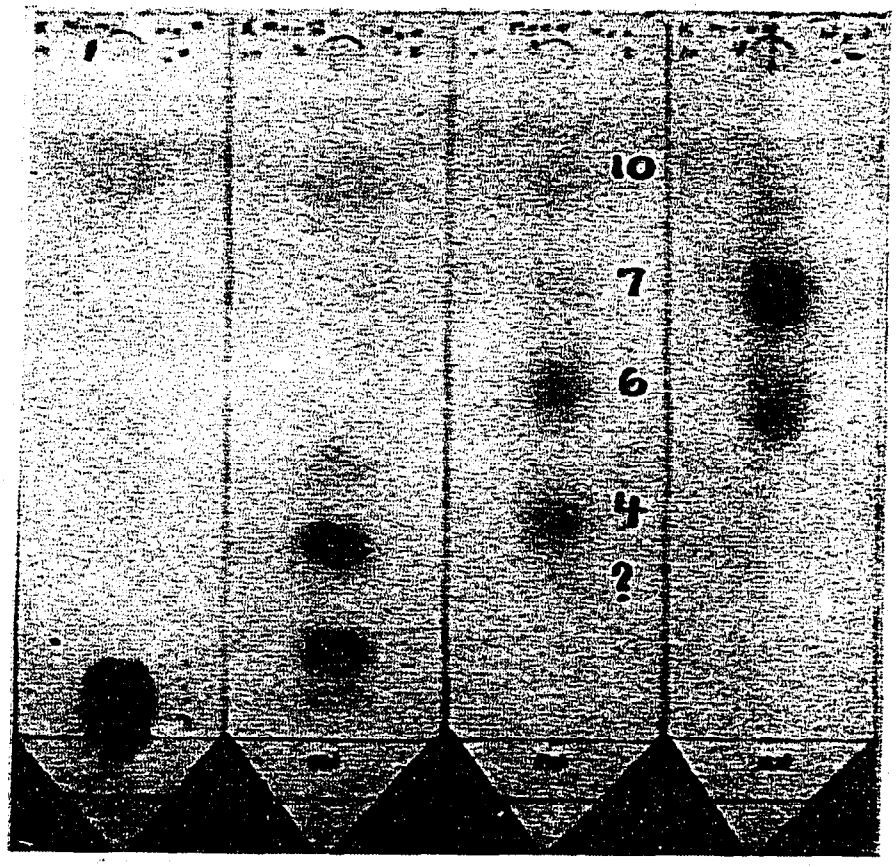

Fig. 5. One hour room temperature run on same extract and paper as Figs. 3 and 4. Ketone 100, methanol left to right 10, 20, 30, 40; water 2; plasmalogen and rhodamin stain. Similar general patterns were obtained on changing the pyridine or acetic acid concentration in their respective solvent systems.

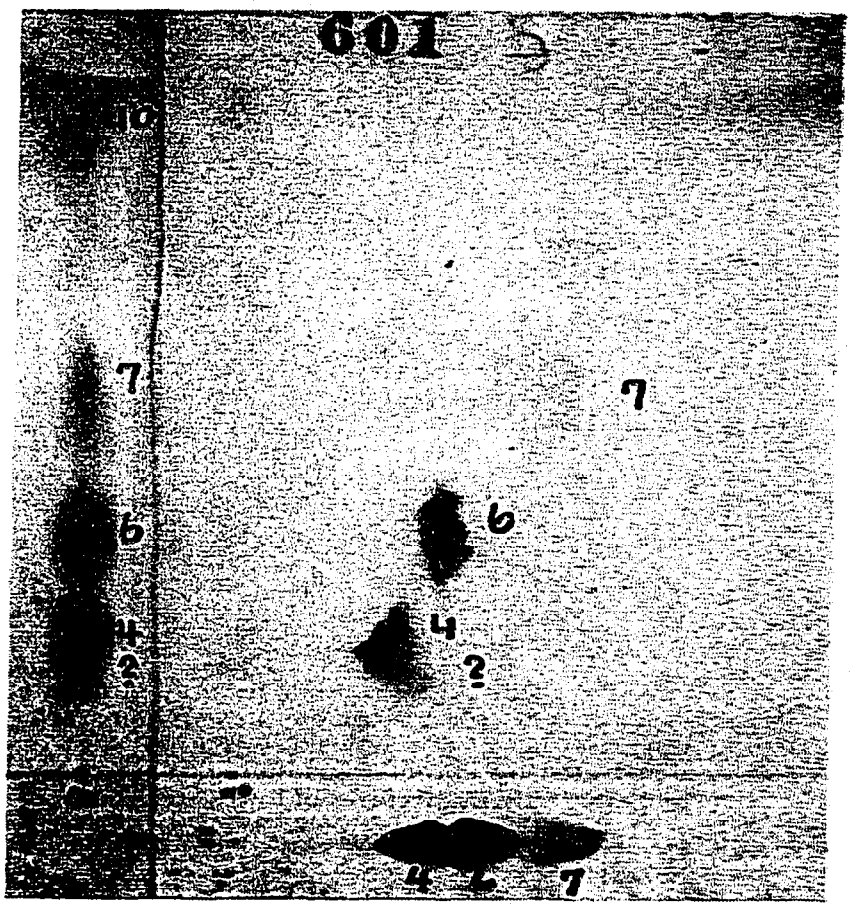

Fig. 6. Two-dimensional chromatogram of same extract and paper as in Figs. 3–5. As oriented here the first dimension was run vertically during 1 h in ketone 100, methanol 40, water 2. After drying, the left-hand strip was cut off, a 25 μl aliquot of extract was added to the bottom strip and the paper again was chromatogrammed (to the right as shown here) during 1 h in ketone 100, pyridine 75, water 6, the left-hand and lower strips representing the separate mobilities. Notice how the material marked ? was resolvable only by the methanol system (see Figs. 2–5); *i.e.*, it had the same mobility as lecithin in the pyridine system. In a similar manner cardiolipin was often shown to be a part of the phosphatidyl ethanolamine spot in the acetic acid system.

methanol 100:10 and chloroform–ethanol 100:15 were similar to the methanol system above, but were much less reproducible even with added water.

A less extensive examination of other ketones (3-methyl-2-butanone, 4-methyl-2-pentanone, 2-pentanone) in various ratios with methanol, pyridine, etc., failed to indicate any general advantage. The higher boiling point of 2,6-dimethyl-4-heptanone was probably responsible for the greater resolving power of this ketone in mixtures as described.

4. *Chromatographic procedure*

Extracts of various animal tissues were prepared according to the method of FOLCH *et al.*[4]; alternately the tissue was freeze-dried and extracted with chloroform–methanol 2:1, 10 ml/100 mg dry weight of tissue; 25 μl of these extracts was generally adequate for analysis. Our extensive experience with cardiac muscle phosphatides[1,2,5] has led us to use extracts of this tissue for control purposes when chromatographing extracts from other sources. Familiarity with the behavior of lecithin and the cephalins in these cardiac muscle extracts as well as their various enzymatic hydrolyses products[2] was far more useful for the interpretation of unknowns than dependence of R_F measurements (which were found to be too variable) and on isolated standard phosphatides which frequently did not keep well even when refrigerated. Similarly the complete battery of spot-tests should be utilized[2] in order to completely characterize the components of each extract. Two-dimensional chromatograms were utilized in order to localize the phosphatides resolved by one solvent system and not by the other; in this way an understanding of the limitations of each solvent system was obtained.

On some occasions, *i.e.*, when the neutral lipid content was high, it was found desirable to prepare the chromatogram in the usual way, but before running in one of the phosphatide solvent systems, the neutral lipids were brought to the top of the paper by a preliminary run in acetone. After drying, the paper was then run in the usual manner. An extension of this technique was to use a long (140 × 290 mm) paper, bring the neutral lipid to the half-way point with acetone, cut the paper in two and chromatogram separately the top half for neutral lipids and the bottom half for phosphatides in the usual manner.

5. *Spot-tests*

Spot-tests useful for characterization of the phosphatides were employed as described earlier[2]. It is essential to emphasize that spot-tests for plasmalogens, after washing briefly in 0.005 M H_2SO_3 to remove residual pyridine, etc., and for unsaturation should be done promptly after completion of the chromatogram in order to avoid the complications arising from air oxidation[6].

RESULTS AND DISCUSSION

The principal results are illustrated by Table I and the figures. In addition it was observed that the mobilities of the lysophosphatides were also markedly influenced by the solvent ratios and were thus not always separable from other phosphatides.

As indicated, it was possible to achieve very adequate resolution of most of the phosphatides providing appropriate adjustments in the solvent ratios were made to meet the situation at hand. In this way we have becomes less dependent on the availability of silicic acid standardized for chromatography or on stable silicate solutions.

TABLE I

DIAGRAM OF THE GENERAL CHROMATOGRAPHIC RELATIONSHIPS OBSERVED IN THE THREE SOLVENT SYSTEMS DESCRIBED

In each case 10 is at the solvent front. The numbers at the right of each column are intended to indicate the variability in the relative mobilities of cardiolipin and phosphatidyl serine, 7 and 5 resp.; as seen from Figs. 2, 3, 4, their mobilities are markedly influenced by the water content of the chromatographic solvent and are thus not always separable from phosphatidyl ethanolamine.

Methanol	*Pyridine*	*Acetic acid*	*Key*	
10	10	10	Neutral lipid	10
		9	Phosphatidic acid	9
8	8	8	Infarct plasmalogen[1]	8
7	7	7	Cardiolipin	7
5 ↓	5 ↓	6 ↑	Phosphatidyl ethanolamine	6
6 ↓	6 ↓	5 ↓	Phosphatidyl serine	5
3 ↓	4 ↓	4 ↓	Lecithin	4
4	3	3 ↓	Lysophosphatidyl ethanolamine	3
2,1	2	2	Sphingomyelin	2
	1	1	Lysolecithin	1

Although we have examined a large number of extracts freshly prepared from many normal tissues from a variety of animal species, we have never observed free-plasmal (higher fatty aldehyde obtained on hydrolysis of plasmalogen) which, in the solvent systems described, would appear at the solvent front[2] and would react immediately with the leuco-fuchsin of the plasmalogen spot-test. Even prolonged storage in the cold did not result in the production of free-plasmal in most of these extracts.

The substance labeled *cardiolipin* was observed in most tissues examined, with the exception of brain and is the subject of continued investigation.

SUMMARY

Techniques for the uniform impregnation of filter paper with silicic acid for the chromatography of phosphatides under controlled conditions are described.

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NOTE ADDED IN PROOF

The influence of water on the chromatographic mobility of phosphatides has also been discussed in a recent paper by J. E. MULDREY, O. N. MILLER AND J. G. HAMILTON, *J. Lipid Research*, 1 (1959)48.