THE SIMULTANEOUS PAPER CHROMATOGRAPHIC SEPARATION OF PHOSPHATIDES, CEREBROSIDES AND SULFATIDES*

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INTRODUCTION

It has proved difficult to study the lipids in myelin because of the complexities involved in their separation. No paper chromatographic system has yet been described which separates phosphatides, cerebrosides and sulfatides simultaneously. This communication describes a new paper chromatographic system in which this is accomplished. This method utilizes silicic acid impregnated paper and a solvent system containing diisobutyl ketone, pyridine and water. Small quantities of lipid mixtures are separated accurately and rapidly.

MATERIALS AND METHODS

Brain (gray matter, white matter, and whole brain) from adult dog, fetal dog, rabbit, monkey, rat and beef and also peripheral nerves from frog, rat and rabbit were obtained immediately after death. The lipids were extracted by one or more of the following methods: (1) by the method of FOLCH *et al.*[1], (2) direct immersion of the tissue in chloroform–methanol, 2:1 v/v (20 vol. of solvent per g of tissue), (3) lyophilization of the tissue and extraction with chloroform–methanol, 2:1 v/v (20 vol. of solvent per g of tissue).

To aid in the interpretation of the chromatograms the following cerebral lipids were used as standards (those prepared in this laboratory were obtained from beef brain):

1. Phosphatidyl ethanolamine and phosphatidyl serine were isolated according to FOLCH's method[2].

2. Sulfatide and strandin samples were (a) obtained from J. FOLCH, and (b) prepared in our laboratory from beef brain as follows: strandin by the method of FOLCH, ARSOVE AND MEATH[3], and sulfatides by the method of LEES, FOLCH, SLOANE-STANLEY AND CARR[4].

3. Sphingomyelin preparation was obtained from E. KLENK AND M. HACK.

4. Cerebrosides were obtained from E. KLENK.

5. "Cephalin B" was prepared as described by BRANTE[5].

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Impregnation of Whatman No. 1 and S & S 2043B filter paper with silicic acid and spot tests were done as described by HACK AND FERRANS[6].

The solvent system utilized consisted of diisobutyl ketone, pyridine and water in the ratio of 100:74:11. (The amount of water in the solvent system was critical and adjustments were necessary with each new batch of silicic acid impregnated paper; usually 9–13 parts of water were satisfactory for our purposes.) Two hundred ml of this mixture were sufficient for a 45 × 15 glass chromatographic cylinder. Five to twenty-five μl of lipid extract were applied to the chromatographic paper with a micropipette, making a spot about 1–2 mm in diameter. The spots were air-dried and the chromatograms developed for 4–12 h at 25°. The chromatograms were again air-dried, the solvent mixture washed out of the paper with two changes of 200 ml of 0.05 *M* H_2SO_3 and then stained for plasmalogens with fuchsin sulfurous acid, washed twice in 200 ml 0.05 *M* H_2SO_3 and counterstained with Rhodamin 6G. Duplicate runs were spot tested for free amino groups, choline and phosphorus.

Counterstaining with cresyl violet in 0.1% aqueous solution for one minute followed by extensive washing in 2% acetic acid was used frequently to demonstrate the metachromatic properties of myelin lipids.

RESULTS

Mobility of the lipids in the solvent system

The following lipids were identified in decreasing order of chromatographic mobility: cholesterol, cholesterol esters, and neutral lipids (as a single spot in the solvent front), cerebrosides, phosphatidyl serine, phosphatidyl ethanolamine, sulfatide, lecithins, sphingomyelin and a lipid close to the baseline which showed characterists of BRANTE's "cephalin B". Strandin was immobile in the system and stayed at the site of application. There was greater resolution of these lipids as the time of development increased.

TABLE I

RESULTS OF SPOT TESTS OF THE SEPARATED BRAIN LIPIDS

FSA—plasmalogen; $-NH_2$—ninhydrin; PHOS.—phosphorus; CHOLINE; META—presence of metachromasia when stained with cresyl violet; RHODAMIN—color of the lipid spots when viewed under U.V. light.

		FSA	*$-NH_2$*	*PHOS.*	*CHOLINE*	*META*	*RHODAMIN*
8	Cholesterol Neutral fat	o	o	o	o	o	yellow
7	Cerebrosides	o	o	o	o	o	light yellow
6	P. Serine	x	x	x	o	o	light orange
	P. Ethanolamine	x	x	x	o	o	light orange
5	Sulfatide	o	o	o	o	x	red-orange
4	Lecithin	x	o	x	x	o	bright yellow
3	Sphingomyelin	o	o	x	x	o	bright yellow
2	Unknown	o	o	x	o	x	red-orange
1	Strandin, etc.	o	o	o	o	x	red-orange

Identification of the lipids

The lipids were identified by comparing their mobilities with standard compounds simultaneously run on the same chromatograms. Individual spot tests for phosphorus, choline and free amino groups provided additional correlating information (see Fig. 1 and Table I).

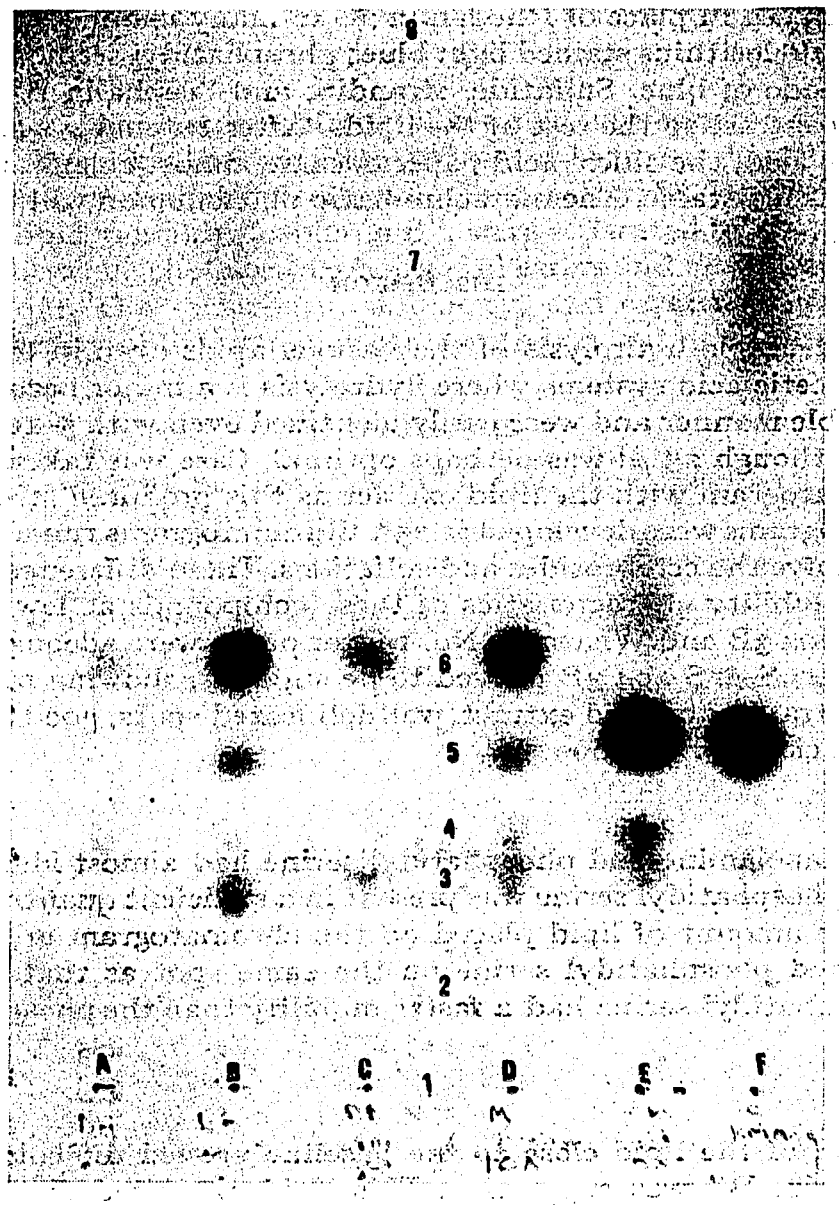

Fig. 1. Spot tests of brain lipids and standard compounds of A. Dog heart; B. Dog brain, white matter; C. Dog brain, gray matter; D. Monkey cerebellum; E. Crude sulfatide; F. Crude keracin.

Rhodamin 6G fluorescence

After staining with Rhodamin 6G, the developed chromatograms were washed several times with 0.05 *M* H_2SO_3 and viewed with an ultraviolet lamp, either wet or after drying. Washing is important to rid the paper of excess dye which sometimes alters the true fluorescing color of the stained lipid compounds. The cerebrosides, sphingo-

myelins and lecithins fluoresced a bright yellow. The sulfatide, strandin and "cephalin B" were a characteristic red-orange; the "cephalin B", however, was less intense due to its quantitative sparcity. When viewed with white light the sulfatides, strandin and "cephalin B" were distinguishable from the other lipids as their spots were red.

Staining with cresyl violet

Cresyl violet was used in place of Rhodamin 6G in many instances. The cerebrosides, sphingomyelins and lecithins stained light blue; phosphatidyl serine and ethanolamine were a darker shade of blue. Sulfatide, strandin, and "cephalin B" were a reddish-purple in sharp contrast to the rest of the lipids. After extensive washing with a 2 % solution of acetic acid, the silicic acid paper became almost colorless while the colors of the lipids remained stable. The metachromatic phenomenon will be amplified later in another communication.

DISCUSSION

The chromatographic system

There was no detectable hydrolysis of the various lipids even in the longer runs as compared with acetic acid systems where hydrolysis is a major hazard. All the lipids behaved in a stable manner and were easily identified even with 5–10 μl of a standard Folch extract, although 25 μl was perhaps optimal. Care was taken not to overload the paper chromatogram with the lipid extract as this produced streaking.

All chromatograms were developed at 25°. Chromatograms run at a lower temperature did not resolve the cerebrosides and sulfatides. These differences were probably caused by the solubility characteristics of these compounds at low temperatures.

Both S & S 204 3B and Whatman No. 1 filter paper were adequate for separation purposes, however, S & S 204 3B proved to be superior, showing minimal streaking with high concentrations of lipid extract, well delineated spots, good staining reaction and good silicic acid impregnation.

Cephalin fraction

Phosphatidyl ethanolamine and phosphatidyl serine had almost identical mobilities. However, when phosphatidyl serine was present in a sufficient quantity (accomplished by increasing the amount of lipid placed on the chromatogram or by placing some chemically isolated phosphatidyl serine on the same spot as that of normal brain tissue), the phosphatidyl serine had a faster mobility than the phosphatidyl ethanolamine.

"Cephalin B"

The identification of the lipid close to the baseline proved difficult. Its likeness to BRANTE's "cephalin B" was suggestive. This lipid exhibited metachromasia with cresyl violet, suggesting a chromogenic group on the molecule, perhaps similar to neurominic acid. Since this lipid also exhibited a positive phosphorus reaction, tentatively it appeared to have the characteristics attributed to "cephalin B".

APPLICATIONS

This chromatographic system has been a useful experimental tool for many problems. We have used this system to advantage in our laboratory in the following ways,

some of which will be elaborated on in a future communication: (1) determining which lipids are responsible for the metachromasia of myelin, (2) demonstrating the cerebrosides in the liver and spleen of a patient with Gaucher's disease, (3) determining the purity of chemically isolated lipids, (4) following the progression of lipids in column chromatography, and (5) providing semi-quantitative lipid information in degenerative and regenerative states of the nervous system.

SUMMARY

A paper chromatographic system utilizing silicic acid impregnated paper and a solvent system using diisobutyl ketone, pyridine and water has been described. Cerebrosides, phosphatidyl serine, phosphatidyl ethanolamine, sulfatide, lecithin, sphingomyelin, and an unknown lipid, possibly "cephalin B", were separated by this system. Strandin remained immobile while cholesterol, cholesterol esters and neutral lipids were located at the solvent front. The sensitivity, simplicity, and rapid lipid analysis are noteworthy. The versatility of the system is commented on and examples of its application are cited.

REFERENCES

1 J. FOLCH, M. LEES AND C. H. SLOANE-STANLEY, *J. Biol. Chem.*, 226 (1957) 597.
2 J. FOLCH, *J. Biol. Chem.*, 146 (1942) 35.
3 J. FOLCH, S. ARSOVE AND J. B. MEATH, *J. Biol. Chem.*, 181 (1951) 819.
4 M. LEES, J. FOLCH, C. H. SLOANE-STANLEY AND S. CARR, *J. Neurochem.*, 4 (1959) 8.
5 G. BRANTE, *Acta Physiol. Scand.*, 15 (1949) suppl. 63.
6 M. H. HACK AND V. J. FERRANS, *Z. Physiol. Chem.*, 315 (1959) 157.