Analysis of Lipids by Spot Tests on Filter-paper Disk Chromatograms

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There is a need in lipid research for a rapid and sensitive method for separating and identifying the lipid components of the mixture obtained by extraction of tissues. For this purpose column chromatography has not been successful (Zechmeister, 1950; Rathmann, 1944; Trappe, 1940*a, b*, 1941), although this technique has been useful in the purification of certain lipids (Thannhauser & Setz, 1936; Klenk, 1942; Hanahan, Turner & Jayko, 1951). Some of the water-soluble hydrolysis products of lipids were recently investigated by means of paper chromatography (Brante, 1949; Levine & Chargaff, 1951*a, b*; Munier, 1951).

We present here a rapid chromatographic technique for the analysis of lipids in quantities of 10^{-2} μ moles or less, using single filter-paper disks. The disk technique has independently been applied by others to the analysis of dyes (cf. Williams, 1947); to cations (Pollard & McOmie, 1951); and to sugars (Bersin & Muller, 1952).

Bevan, Gregory, Malkin & Poole (1951) and Hecht & Mink (1952) have recently reported paper-chromatographic techniques for the analysis of phosphatides.

METHODS

The tissue extract

Extraction of lipids from freeze-dried tissue can be effected as described previously for blood and plasma (Hack, 1947). Because cephalins, present in these extracts, are firmly adsorbed on filter paper it is necessary to use alundum or sintered-glass thimbles in order to avoid selective loss of these phosphatides. For this reason, extracts prepared by other procedures should not be filtered through filter paper but should be clarified by centrifugation or by filtration through sintered glass.

A CHCl_3 :methanol (4:1, v/v) extract of freeze-dried tissue may in general be presumed to contain the total lipid; a benzene extract may be presumed to contain all lipid not bound to protein. This notion is based on the observation that polar solvents are required to extract lipid from lipoprotein (cf. Chargaff, 1949; Hack, 1947). Acetone extracts neutral fat, cholesterol and certain other non-phosphatide lipids; the lipid remaining in the tissue can be removed by CHCl_3 :methanol and thus represents the acetone-insoluble fraction. In this way a convenient preliminary fractionation can be effected to precede chromatographic analysis.

Experience has shown wet tissues to be less distinctly fractionated according to this scheme, possibly because of the influence of tissue water and water-soluble compounds on lipid solubilities.

Preparation of the chromatogram

The chromatograms were made with Schleicher & Schuell no. 576 or with Whatman no. 1 filter-paper disks (5.5 cm.) previously extracted with CHCl_3 :methanol (4:1, v/v) and dried. The centre of the filter paper was indicated by a dot made with a pencil. With the filter paper supported on the rim of a 50 mm. Petri dish, 25 μ l. of a CHCl_3 :methanol, benzene or acetone solution containing 10^{-2} μ moles of lipid were added slowly with a Carlsberg pipette to the centre of the paper, confining the diameter of the sample spot to about 8 mm.; the solvent was evaporated by a current of nitrogen. Portions of CHCl_3 :methanol (25 μ l.) were then added until the lipid was spread to a ring 10 mm. in diameter (indicated by a pencil mark); two such portions usually sufficed. Finally, 25 μ l. portions of the desired eluant were added at such a rate that the diameter of the wet spot increased about 2 mm./sec.; three such portions were generally sufficient to effect as complete separation of the lipid components as could be accomplished by the eluant chosen. The total time required for these operations was of the order of 5 min. When the eluant had been evaporated by a current of N_2 , appropriate tests (see below) showed sharply defined concentric rings of the separated lipids. Often more complete separation could be effected by using two or more eluants.

It was frequently found desirable to cut the filter-paper chromatogram into two or more sectors and to test each piece with a different reagent in order to characterize the sample fully (cf. Figs. 1 and 2).

We have found it unnecessary to control the atmospheric conditions of the laboratory beyond avoiding strong air currents which may evaporate the solvent too rapidly.

An example. A 0.2 ml. sample of blood serum from a fasting human subject was freeze-dried and extracted with 1 ml. of CHCl_3 :methanol at room temperature during 1 hr. The extract was then collected by filtering through a small medium-porosity sintered-glass filter. Two 25 μ l. portions of this clear, pale-yellow extract were applied to a filter paper as described above. 25 μ l. of CHCl_3 :methanol were then added, bringing the lipid to a ring 10 mm. in diameter. After removal of the CHCl_3 :methanol by a current of N_2 , three 25 μ l. portions of methanol were applied, not allowing the diameter of the wet spot to exceed 30 mm. After drying with N_2 the chromatogram was cut in half; one half was tested for choline by the dichromate-haematein method and the other treated with Nile blue. The result is schematized in Fig. 1*A*. A better separation was effected by eluting first with four 25 μ l. portions of acetone to diam. 40 mm., drying, and then eluting to diam. 30 mm. with methanol as shown in Fig. 1*B*.

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In a similar manner a mixture of purified lipids can be separated as indicated in Fig. 2.

Eluants. The following organic solvents, dried by distillation over anhydrous CaSO_4 , were found to be most generally useful as eluants because of their differential solvent action: acetone, benzene, CHCl_3 and methanol. Others, which could probably profitably be employed are pyridine, ethyl acetate, carbon disulphide and dichloro-ethylene.

Fig. 1. Chloroform-methanol (4:1, v/v) extract of human blood serum eluted to *a* (A and B) with CHCl_3 :methanol, to *b* (A and B) with methanol and to *c* (B) with acetone. The left half was treated for the detection of choline by the dichromate-haematein technique and the right half was treated with Nile blue. The black ring indicating choline is shown as a solid line in the figure; the red Nile-blue ring as a dotted line and the blue Nile-blue ring as a dashed line.

Fig. 2. Mixture of phosphatidyl serine (*a*), hydrolecithin (*b*), and glyceryl palmital (*c*) eluted by methanol to distance *c*. The five sectors were treated separately for the detection of: 1, amine; 2, choline; 3, acetal; 4, phosphate; 5, unsaturation.

Spot tests

Tests for choline lipids. (1) When the chromatogram is immersed, without previous hydrolysis, in aqueous 0.05M-reinecke salt for 2 or more hours at room temperature, a pink ring appears at the site of the choline lipid (cf. Baer & Kates, 1950; Hack, 1946). Thorough rinsing in water removes the excess reagent and when dry the chromatogram may be retained as a permanent record. (2) Immersion for 1 min. in 0.0001M- I_2 in aqueous KI at room temperature produces a transient yellow-brown colour (cf. Brante, 1949). (3) Reaction of chromatograms with aqueous 0.1M $\text{K}_2\text{Cr}_2\text{O}_7$ at 60° for 1 hr. results in a faint brownish ring at the site of the choline lipid. This ring can be intensified by reacting with

a freshly prepared solution of haematein (a mixture of 25 mg. haematoxylin, 50 ml. water and 0.2 ml. 30% (w/v) H_2O_2 heated to 60° for 10 min.) for 10 min. at 60°. The more prominent blue-grey chromium lake of haematein appears at the site of the brownish ring (cf. Baker, 1947). Excess reagent must be removed with water at each step. Similarly, aqueous 0.005M-phosphomolybdic acid (cf. Levine & Chargaff, 1951*a*, *b*) can be used; in this case a blue-lavender haematein lake is formed.

Test for amine lipids. Wetting the filter paper with aqueous 0.005M-ninhydrin and drying for 15 min. at 105° results in a lavender ring at the site of lipids with a free NH_2 group.

Test for acetal lipids (plasmalogens). Hydrolysis of the acetal bond in the presence of aqueous 0.005M- HgCl_2 at room temperature for 1 min. exposes the carbonyl group for reaction with fuchsin-sulphurous acid or with aqueous 2:4-dinitrophenylhydrazine. The excess fuchsin-sulphurous acid is removed with aqueous 0.05M- SO_2 and the 2:4-dinitrophenylhydrazine with water (cf. Hack, 1952).

Test for phosphoric ester. Phosphate is detected as a blue ring when chromatograms are exposed to acid ammonium molybdate followed by H_2S according to the method of Hanes & Isherwood (1949). In this test sphingomyelin reacts more slowly than glycerophosphatides.

Test for cholesterol. Exposure of chromatograms to a 1:1 (v/v) mixture of acetic acid and H_2SO_4 (cooled to 20°) results in the appearance of a pink ring at the site of cholesterol.

Test for glycolipids. Glycolipids (cerebrosides, etc.) can be detected by oxidation with aqueous periodic acid and visualization of the resulting aldehyde by fuchsin-sulphurous acid as shown by Morrison & Hack (1949). The acetal lipids also give this test but can be differentiated from glycolipid by the plasmal reaction. However, some acetal lipids have elution characteristics similar to the glycolipids and have existed resolution by the eluants mentioned.

Test for unsaturation. Lipids containing unsaturated fatty acids and aldehydes appear as a brown to black ring after exposure for 5 min. to OsO_4 vapours.

Other tests. Although the mechanism of their reaction is not known, both aqueous 0.01M-Biebrich scarlet and 0.01M-Nile blue in 0.1M- H_2SO_4 were also used. Biebrich scarlet was found to be a useful stain for choline lipid. In general, a blue colour with Nile blue seems to indicate a strong acid function as in cardiolipin, phosphatidyl serine, etc., and a pink colour, a strongly lipophilic compound as in free fatty acid, fatty aldehyde, neutral fat, etc.

RESULTS

Some of the results with a variety of purified lipids appear in Table 1. In addition to the observations given in this table, it was found that with iodine, a transient yellow-brown ring appeared at the site of phosphatides, which persisted longer when the compound contained choline. Both the phosphatidyl serine and the diposphoinositide were shown to contain a material eluted by acetone and staining pink with Nile blue, presumably free fatty acid. In addition, the phosphatidylserine contained a second ninhydrin-positive substance (not the intact phosphatide) elutable by acetone. We believe these

Table 1. *Spot-test reactions of various lipids*

(Compounds 1 and 12 were obtained through the generosity of E. Klenk; 3, 5 and 7 from S. J. Thannhauser; 4 and 8 from E. Baer; 6 and 11 from M. C. Pangborn and the Sylvania Chemical Co.; 9, 10 and 15 from J. Folch; 19 was a Hormel Foundation product; 20 and 21 from Armour and Co.; 22, an Eastman Kodak product; 2, 13, 14, 16, 17, 18 were prepared by the author. Preparations 2, 13, 14 were described earlier (Hack, 1946, 1947). The ganglioside (16) was isolated from human brain by the procedure of Klenk (1941) and the glycerylpalmital (18) was synthesized according to Bersin, Moldtman, Nafziger, Marchand & Leopold (1941). The cholesterol (17) was isolated from an acetone extract of human brain and was purified by repeated recrystallization from methanol. For experimental details see text. +, Positive; 0, negative.)

Compound	Reinecke salt	Potassium dichromate-haematein	OsO ₄	Ninhydrin	Plasmal	Phosphate
1. Sphingomyelin (brain)	+	+	+	0	0	+
2. Sphingomyelin (brain)	+	+	+	0	0	+
3. Sphingomyelin (lung)	+	+	+	0	0	+
4. Dipalmityllecithin (synthetic)	+	+	(slight)	0	0	+
5. Dipalmityllecithin (lung)	+	+	0	0	0	+
6. 'Lecithin' (beef-heart)	+	+	+	0	+	+
7. Acetal phosphatide (brain)	0	Brown (slight)	+	+	+	+
8. Phosphatidyl ethanolamine (synthetic)	0	Brown (slight)	(slight)	+	0	+
9. Phosphatidyl serine (brain)	0	Brown (slight)	+	+	0	+
10. Potassium diphosphoinositide	0	0	.	.	0	+
11. Cardiolipin (beef-heart)	0	0	+	0	0	+
12. Phrenosin (brain)	0	0	0	0	0	0
13. Phrenosin (brain)	0	0	0	0	0	0
14. Kerasin (brain)	0	0	0	0	0	0
15. Sulphatide (brain)	0	Brown (slight)	.	0	0	.
16. Ganglioside (brain)	0	.	.	+	0	.
17. Cholesterol (brain)	.	0	0	.	.	0
18. Glycerylpalmital (synthetic)	.	0	0	.	+	0
19. Oleic acid	.	0	+	.	0	0
20. Palmitic acid	.	0	0	.	0	.
21. Stearic acid	.	0	0	.	0	.
22. Choline HCl	+	+	.	0	0	.

observations to indicate contamination by products of auto-oxidation.

The beef-heart 'lecithin' was separable from a ninhydrin-positive plasmalogen with methanol. By elution with chloroform, benzene, or acetone, this 'lecithin' yielded two choline components, both plasmalogens, hitherto undescribed. Preliminary experiments have shown glycerylphosphorylcholine and higher fatty aldehyde to be present in good yield after hydrolysis in 50% (v/v) aqueous ethanol containing 0.001 M-HgCl₂ during 3 hr. at 20°. A detailed analysis of these choline plasmalogens is being undertaken. To avoid confusion the original name given to this substance (cf. Pangborn, 1941, 1945) is retained (in inverted commas), although our experiments indicate that the major component probably could be considered to be acetal-phosphatidyl choline. The multiplicity of plasmalogens has recently been demonstrated by Lovern (1952) who applied the technique of countercurrent distribution to the separation of phosphatides from ox brain.

The lecithins were separable from sphingomyelins

by elution with chloroform and from kephalins by methanol. Sphingomyelins were eluted more rapidly by methanol than were cerebrosides. Plasmalogens were separable by acetone and by methanol.

Exposure of chromatograms to 0.1 M-KOH at room temperature for 20 hr. followed by thorough washing in water and extraction by acetone left only the sphingomyelins, which were demonstrable with dichromate-haematein.

In a preliminary study, several animal tissues (heart, spleen, anterior pituitary, lung, adrenal, kidney, mammary gland, spinal cord and blood serum) were examined by this technique; the extract from 1 mg. of freeze-dried tissue was more than sufficient for analysis. By the use of eluants, each of these tissues was shown to contain two or more plasmalogens, one or more of which was acetone-soluble and therefore probably not phosphatide. Also, by assay of benzene and chloroform-methanol extracts (cf. Hack, 1948), it was possible to estimate the proportion of protein-bound lipids in these tissues.

DISCUSSION

It should be emphasized here that the eluants used and the order of their use must be empirically determined for each tissue so as to obtain the best separation of the lipids in a given extract and that our procedure is to be regarded only as preliminary and to be modified as required.

R_f values (in terms of radial distance) were not determined for any of the compounds studied and are not requisite for its use as an empirical tool as described. This technique has served as a reliable test for the homogeneity of isolated lipids, and in some cases has permitted characterization of the contaminant.

The small quantities of tissue required made it practical to use the technique as an adjunct to histochemical studies of lipids, especially with plasmalogens (Hack, 1952), as it affords a means of verifying the presence of the lipid in question. Efforts are being made to apply some of the tests described to the *in situ* identification of lipids in cells and portions of cells. However, great caution must be exercised because some of the colour reactions

described above are given by a variety of non-lipid nitrogenous substances (Guggenheim, 1920) occurring as constituents of proteins.

SUMMARY

1. A technique is presented for the examination of lipids in quantities of 10^{-2} μ moles by means of spot tests applied to filter-paper chromatograms. This technique permits the separation and characterization of lipid mixtures both in tissue extracts and in separated lipids.

2. It has become evident that several plasmalogens, detected by their elution characteristics, are present as constituents both of separated lipids and of tissues.

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