Two-dimensional chromatography on silica gel-loaded paper for the microanalysis of polar lipids

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ABSTRACT Two-dimensional chromatography on commercially available silica gel-loaded paper for the microanalysis of polar lipids from various tissues is described. All common phospholipids and their lyso derivatives can be reproducibly separated. As many as 22 lipid components were separated on a single chromatogram. Improved methods for staining lipids and for determining phosphorus in the chromatographic spots are reported.

KEY WORDS paper chromatography · two-dimensional · silica gel-loaded paper · phospholipids · P determination · polar · lipids · cartilage · bone · Rhodamine 6G staining · Dragendorff stain

Unidimensional chromatographic procedures which use thin-layer chromatography (1–5), silica gel impregnated papers (6–9), or formaldehyde-impregnated papers (10–12) yield a complete separation of polar lipids only if the lipid mixture is simple. Recently Skidmore and Entenman (13) and Abramson and Blecher (14) have employed two-dimensional thin-layer chromatography, while Letters (15) and Marinetti (16) have employed two-dimensional chromatography with commercially available silica gel-loaded papers for the separation of phospholipids. Although these procedures have permitted more complete separations than one-dimensional systems, all have one or more disadvantages. In most of these methods (13, 14, 16) the separation of lyso derivatives has not been thoroughly explored. In the method (15) where this has been dealt with, two types of paper were required, one of which had to be hand-made.

This report concerns several two-dimensional chromatographic procedures which utilize commercially available silica gel-loaded paper for the separation of polar lipids. Phospholipids, their lyso derivatives, and various polar lipids not containing phosphorus are separated. The methods are simple, relatively insensitive to variations in humidity, temperature, and degree of activation, and are very reproducible. Compared with Marinetti's system (16) which used the same commercially available paper, larger amounts of lipid may be applied without overloading. Complex mixtures of polar lipids from a wide variety of tissue extracts have been separated into as many as 22 lipid components on a single chromatogram. A number of new lipids have been detected. The procedures were developed for the analysis of cartilage and bone lipids of which only a few milligrams of total lipid were available.

MATERIALS AND METHODS

Tissues were obtained as soon as possible after death (2–15 min) and those rich in lipid (brain, liver, heart muscle, and bone marrow) were extracted fresh with chloroform–methanol 2:1 according to Folch, Lees, and Sloane Stanley (17). Fetal cartilage and bone were frozen and stored with solid carbon dioxide until used. These were then dissected, washed free from blood with 0.9% NaCl, lyophilized, extracted with chloroform–methanol 2:1, demineralized with 0.5 M EDTA (pH

Abbreviations: PA, phosphatidic acid; PS, phosphatidyl serine; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PI, phosphatidyl inositol; DPG, diphosphatidyl glycerol; Sph, sphingomyelin; Ce, cerebrosides; CeS, cerebroside sulfates; LPS, lyso-PS; LPE, lyso-PE; LPC, lyso-PC; LPI, lyso-PI; FFA, free fatty acids; DEAE, diethylaminoethyl. Solvent ratios are v/v.

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TABLE 1 Composition of Chromatographic Solvent Systems

Solvent System	Usage	Development Time	Chloroform	Methanol	Diisobutyl Ketone	Acetic Acid	Pyridine	Water	0.5 M pH 10.4 Ammonium Chloride Buffer	Comments
			Volume of Each Reagent Per Chamber*							
		hr				*ml*				
1	First dimension	2.75	23	10	45	25	—	4	—	General use.
2	First dimension	2.50	45	15	30	20	—	4	—	General use, wider separation of LPC, Sph, & PC, less separation of PS & PC.
3	Second dimension	1.50	30	17.5	25	—	35	—	6	General use.
4	Second dimension	1.50	30	25	25	—	35	—	8	General use, wider separation of LPS & Sph, less separation of PC & LPE.
5	Second dimension	1.50	30	25	25	—	35	8	—	Separation of LPE from PC & PI, PS tends to streak.

* Three-liter amber wide-mouth bottles were used as chromatographic chambers.

8.0), dialyzed, lyophilized, and reextracted with chloroform–methanol. They were finally extracted with chloroform–methanol–concd HCl 200:100:1 according to Folch (18). The acidic extract was neutralized with dry $NaHCO_3$ and filtered. Only the final acidic extracts of the fetal tissues are described here because they contained several new lipids. All extracts were concentrated to dryness under a reduced pressure of nitrogen and then purified from nonlipid contaminants on Sephadex columns (19).

Lipid Standards

Purified lipids obtained from Applied Science Laboratories Inc., State College, Pa., were used to identify sphingomyelin, phosphatidyl choline, phosphatidyl serine, lysophosphatidyl choline, lysophosphatidyl ethanolamine, cerebrosides, and cerebroside sulfates. Phosphatidyl ethanolamine, phosphatidyl inositol, and diphosphatidyl glycerol were prepared from rat liver lipids by chromatography on DEAE cellulose and silicic acid columns according to Rouser, Kritchevsky, Heller, and Lieber (20). Other lyso derivatives were prepared by mild alkaline degradation of parent phospholipids according to the method of Marinetti (8). Phosphatidic acid was prepared from calf heart lecithin according to the method of Davidson and Long (21) and purified by chromatography on silicic acid and DEAE cellulose columns.

Chromatographic Procedure

Whatman SG-81 silica gel-loaded paper, available from H. Reeve Angel & Co., Inc., Clifton, N.J., in 46 × 57 cm sheets, was cut into nine equal-sized sheets, 15.3 × 19.0 cm. Papers were stored in polyethylene or other air-tight containers to prevent adsorption of moisture and contaminants from laboratory air.

Large-mouth 3-liter amber round bottles were used as developing chambers. Larger vessels required longer development time but yielded no better resolution. Routinely, 100 ml of each solvent system was used for each chamber.

The composition and usage of the various solvent systems are presented in Table 1. Solvents were redistilled before use even though of reagent grade. Diisobutylketone was redistilled under vacuum so that thermal decomposition was minimized and then stored under nitrogen. All systems were made fresh daily, although they could be used the following day with little change in the quality of separation. After addition of the solvent mixtures, the chambers were shaken vigorously and allowed to stand for 5–10 min before the papers were introduced.

For general use solvent 1 (Table 1) was used for the first dimension. Alternatively, to achieve somewhat greater separation of LPC, Sph, and PC, we used solvent 2 for the first dimension. For general use in the second dimension, solvent 3 was employed. Alternatively, solvent 4 was used if it was desired to effect almost complete separation of LPS from Sph; however, this solvent failed to separate LPE from PC. Another alternative for the second dimension was solvent 5. This system gave complete separation of LPE from PC, but PS tended to streak.

Tissue lipids containing 5–15 μg of P were applied to the paper, with drying under nitrogen, as a 5 mm diam-

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eter spot in one corner, 2 cm from each edge. The first solvent was allowed to ascend in the long direction of the paper, which was rolled into a cylinder held together with stainless-steel wire clips. No prior equilibration of the papers in the saturated atmosphere was necessary. Between runs the papers were dried at room temperature in a hood for 30 min in dry or cool weather, or for 5–10 min in a desiccator under high vacuum after an initial 10 min drying in the hood in warm, humid weather. Chromatography was done in an air-conditioned laboratory; nevertheless, in tests outside the laboratory in hot humid weather, good results were obtained if papers were dried in a desiccator under high vacuum for 5 min, after the lipids had been applied but before the paper was introduced into the chamber. Overnight drying of the paper between the two runs was avoided because lipids tend to degrade and cause streaking.

Detection of Lipids

Rhodamine 6G. The method was basically that of Marinetti (8); however, certain changes were required to insure success with the stain in the methods here described. After the usual preliminary rinsing with distilled water, the papers were rinsed further with 0.1 M acetic acid and again with distilled water before being stained with Rhodamine 6G as described by Marinetti. The rinse with weak acid restored the normal staining properties of acidic lipids which were altered by the alkaline, pyridine-containing solvent systems. After the staining, the chromatograms were rinsed 3 times more with deionized water (to reduce P background), allowed to drain briefly while they were still in the tray, examined in the dark under 366 mμ UV light, and hung to dry in a hood. Papers were not allowed to dry in the tray, otherwise the lyso derivatives tended to diffuse out from originally compact spots and overlap adjacent areas. Papers were viewed under UV light several times during the course of drying—when wet, when slightly damp, and again when fully dry. Neutral lipids generally appeared yellow or pinkish under UV light; acidic lipids appeared purple or bluish. Because the appearance of the spots under UV light changed and the spots sometimes disappeared upon complete drying, it was found useful to circle the spots with a dull pencil and indicate their colors.

Ninhydrin. The method was essentially that of Marinetti (8) except that 0.5% ninhydrin in acetone–pyridine 75:25 was used. As Marinetti suggested, heating should be avoided because the purple color is then less intense and less stable than if it is allowed to develop at room temperature.

Dragendorff Reagent. This was adapted for paper chromatography from the procedure of Wagner, Hörhammer, and Wolff (3) for the detection of choline lipids on thin-layer chromatography. The brick-red precipitate which formed on mixing the reagents was allowed to settle for 5 min and filtered off; the yellow-orange solution was used. Papers were washed twice in 0.5 M acetic acid, stained in the Dragendorff solution for 10 min, and rinsed briefly with water. Choline-positive spots were pinkish-orange against a yellow background. Papers must be washed with acetic acid, otherwise a general brick-red precipitate forms on the paper and obscures the choline-positive areas. This stain was not used if the chromatograms were to be analyzed for phosphate.

Phosphorus Analysis

This was adapted for lipid P analysis from the method of Martin and Doty (22). Perchloric acid was substituted for sulfuric acid to facilitate lipid digestion and a more concentrated stannous chloride solution was used. A range from 0.2 to 3.0 μg of P per tube was used, amounts higher than 3.0 μg per tube being diluted before color development (see below). Absorbancy per μg of P in the tube (11 mm light path) was 0.162 ± 0.004.

Spots from chromatograms were cut out, along with several blank areas (each 40–60 mg) well removed from known or suspected spots. These were weighed and then digested until colorless (ca. 1 hr) in glass-stoppered tubes in a 180°C sand bath with 0.5 ml of 70% perchloric acid containing 1 g of ammonium molybdate per liter. The weight of the paper bearing the spots was usually 10–60 mg; when it exceeded 70–80 mg, it was advisable to divide the spot between two tubes, otherwise digestion was slow. After cooling, 1.5 ml of water, 0.25 ml of 10% ammonium molybdate, and 1.5 ml of benzene–isobutanol 1:1 were added; the tubes were stoppered, shaken vigorously 40 times, and centrifuged briefly to insure complete separation of the phases. Aliquots (1 ml) of the upper phase were transferred to colorimeter tubes and the volume was made up to 3.0 ml with absolute ethanol–concd sulfuric acid 96.8:3.2. An automatic dilutor such as that supplied by Labindustries, Berkeley, Calif., was very useful for this step. For tubes in which the intensity of the yellow color in the upper phase was greater than that of the highest P standard, an appropriately smaller aliquot was taken, the volume difference being made up with the ethanol–sulfuric acid solution. Care was taken not to include the lower phase in the aliquot of upper phase, otherwise the reduction with stannous chloride was incomplete. The aliquot and diluent were mixed, 0.5 ml of stannous chloride reagent (10% $SnCl_2$ in concd HCl, stock; diluted 1:100 with N sulfuric acid just before use) was added, and the contents of the tubes were mixed thoroughly. After 5 min the absorbancy of the tubes was read at 725 mμ.

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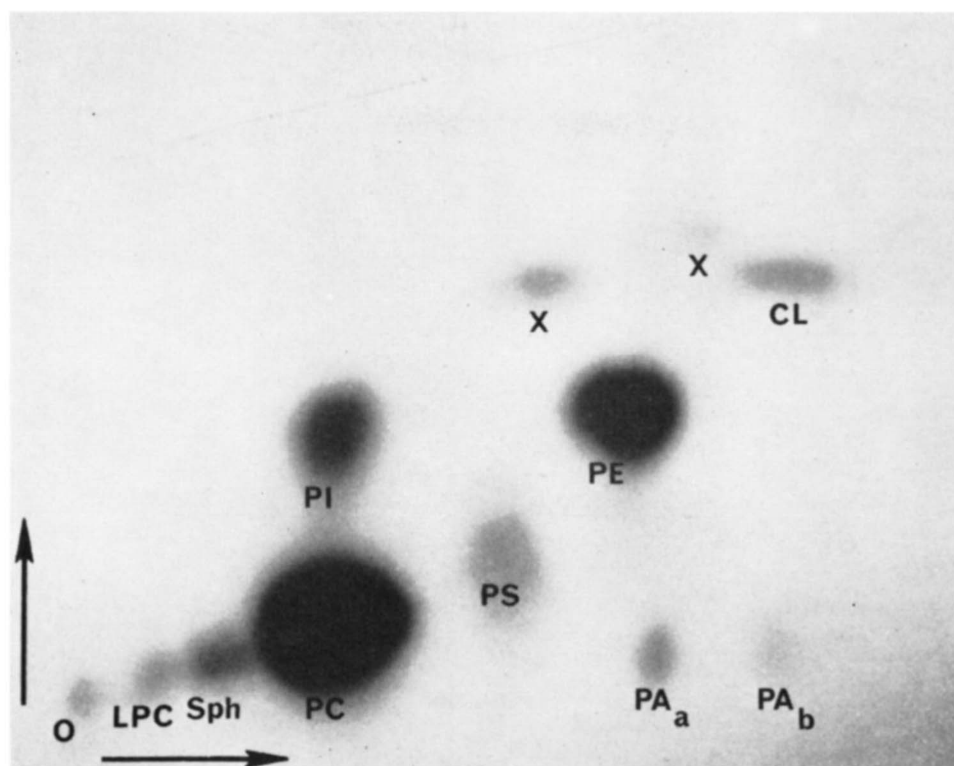

FIG. 1. Autoradiograms of chromatograms of ^{32}P-labeled rat kidney phospholipids. Autoradiograms of lipids extracted 2 and 13 hr after injection of ^{32}P are superimposed in order that both rapidly and slowly labeled lipids may be presented. Solvents 1 and 3 were used to develop the chromatograms. The long exposure required to demonstrate minor phospholipids resulted in some fogging around areas of heavily labeled lipids.

Correction was made for the P content of the paper, which averaged 0.0026 μg/mg.

Autoradiography of ^{32}P-Labeled Lipids

Two 50-g albino rats were injected intraperitoneally with 1 mc of carrier-free phosphate-^{32}P in 0.9% saline. The rats were sacrificed 2 and 13 hr after injection. The liver and kidney lipids were handled as previously described in the preparation section. Approximately 550 and 600 μg of kidney and liver lipids respectively, per chromatogram, were used for autoradiography. Chromatograms of labeled lipids were exposed to Kodak No-Screen X-Ray film for 3 and 6 days for the 13 and 2 hr extracts.

RESULTS

Fig. 1 shows autoradiograms of two-dimensional chromatograms of kidney lipids extracted 2 and 13 hr after ^{32}P injection. Solvents 1 and 3 were used to develop both chromatograms. Fig. 2 shows autoradiograms of 2 hr and 13 hr chromatograms of liver lipids from the same rats; in this case solvents 2 and 4 were used. Two-hour and 13-hr autoradiograms were superimposed in order to present both rapidly and slowly labeled phospholipids on each figure. In Fig. 3 is presented a map of superimposed two-dimensional chromatograms of lipids from various mammalian tissues, developed with solvents 1 and 3. The legend to Fig. 3 presents the appearance of the Rhodamine 6G stained lipids under 366 mμ light at various stages of dryness.

As is evident from the figures, the methods permit complete separation of the major polar lipids and their lyso derivatives, and also resolve many additional uncharacterized lipids. Partial overlap occurred between LPE and PC, and LPS and Sph, with solvent pair 1 and 3. However, virtually complete separation of LPE from PC and PI, and LPS from Sph, was achieved by using solvent pairs 2 with 5, and 2 with 4, respectively. Even so, reasonable quantification of partially overlapped lipids can be achieved by analyzing the overlapped area separately and calculating the amount of lipid P in each component on the basis that area × concn = total P.

Table 2 (p. 550) presents data on the composition of various mammalian tissue lipids. The residual lipids from preextracted demineralized calcifying tissues are also presented because of their unusual composition. Where they are comparable, the results are in agreement with the published values of Dawson (23), Dawson, Hemington, and Davenport (24), and Letters (15).

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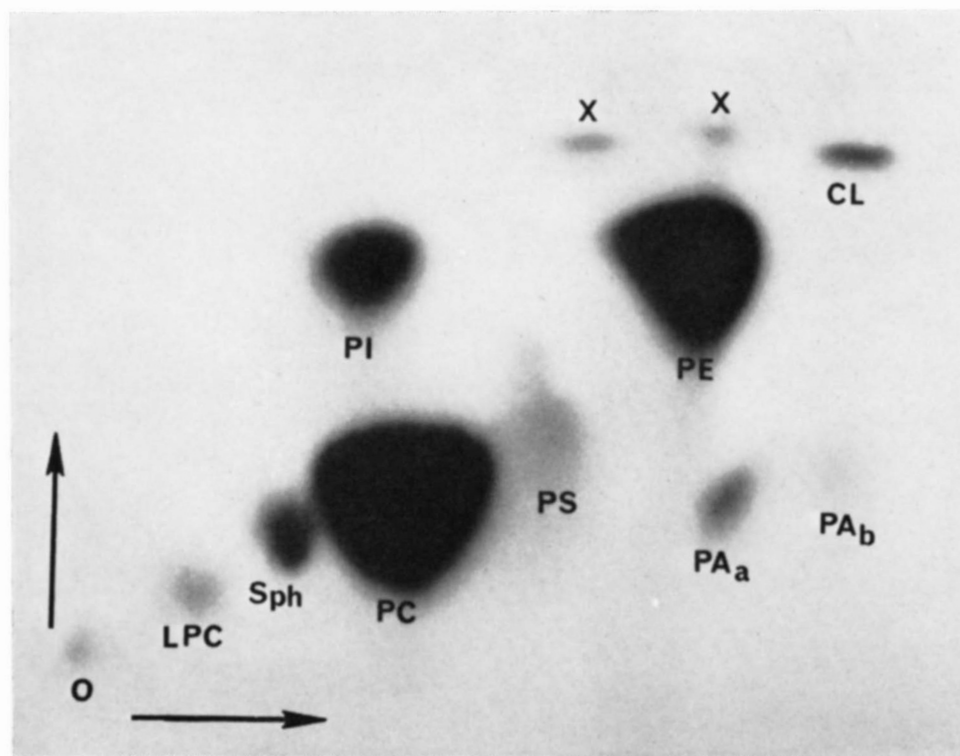

FIG. 2. Autoradiograms of chromatograms of ^{32}P-labeled rat liver phospholipids, handled as described in Fig. 1, except that solvents 2 and 4 were used to develop the chromatograms.

DISCUSSION

The present methods offer certain advantages over thin-layer chromatography for routine quantification of phospholipids. The papers require no preparation and are uniform in quality, and the stained chromatograms can be conveniently stored in folders. For quantification of lipid P the chromatographic spots are then cut out, weighed, and transferred to digestion tubes. In addition, the methods appear to be less sensitive to variations in temperature and humidity.

The main advantages over Marinetti's (16) two-dimensional method with the same Whatman SG-81 paper are that wider separations of most of the lipids are achieved and larger amounts of lipid may be applied. In our hands, Marinetti's system was overloaded when 10–15 μg of lipid P were applied, whereas this amount of P is needed for an accurate analysis of the separated components.

Although Letters' (15) procedure seems to give as good or better resolution of phospholipids and their lyso derivatives, it is less useful for routine analysis because it requires two types of paper and more involved technique.

The solvent systems employed by these methods are either original or extensively modified from any reported in the literature. Solvent systems 1 and 2 were based on Marinetti's (7) original diisobutylketone–acetic acid–water system. However, it was found that by the addition of various amounts of chloroform and methanol, considerably wider separations of PS from PC and PE, and especially greater resolution of LPC, Sph, and PC were achieved. The resultant systems are less sensitive to variations in temperature and humidity, probably because the addition of methanol increases the capacity of the mixtures to retain water.

The development of the solvent systems for the second dimension was based on the principle that alkaline systems should separate a number of lipids not resolved by acidic ones. Early studies indicated that pyridine–diisobutylketone–water 50:50:7 separated a number of lipids not resolved in diisobutylketone–acetic acid–water, but failed to separate CeS from PI or PA from DPG, and tended to cause streaking of PS. Substitution of increasing concentrations of ammonium chloride buffer for water caused progressive reduction in the streaking of PS and increasing retardation of PI and PA. These systems, however, tended to demix when used after development in the first dimension, forming a troublesome secondary solvent front which distorted the shape of lipid spots migrating near to it. Addition of chloroform and methanol greatly reduced the demixing tendency.

In a number of tissues (brain, liver, kidney, and bone marrow) PA often appeared as a double spot (Fig. 3,

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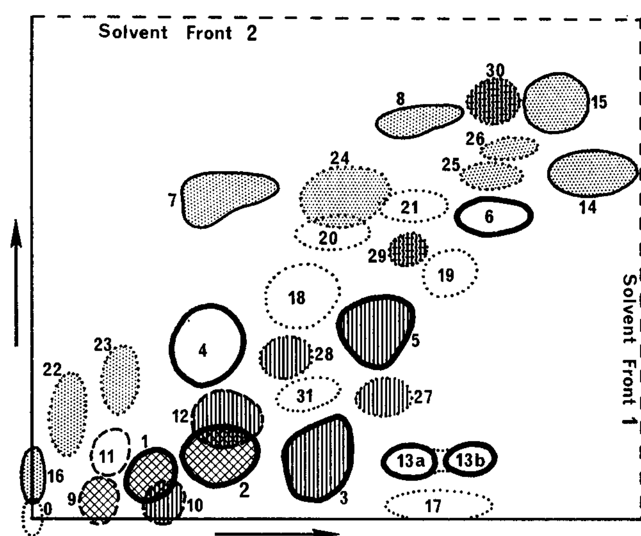

FIG. 3. Map of two-dimensional chromatograms of lipids from chloroform–methanol and acidified chloroform–methanol extracts of various mammalian tissues. Solvents 1 and 3 were used to develop the first and second dimensions, respectively. Heavy outlines, common phospholipids; dashed outlines, lysophospholipids; lighter solid outlines (e.g. No. 7), identified nonphospholipids; and dotted outlines, uncharacterized lipids. Vertical hatching, ninhydrin-positive lipids; cross-hatching, choline-positive lipids; and stippled, nonphospholipids. The identity of individual lipid spots and their colors under UV light (366 mμ) on wet, slightly damp, and dry chromatograms respectively are:

0 Origin
1 Sphingomyelin (Pi, Y-Pi, Y)
2 Phosphatidyl choline (Y-Pi, Y, Y)
3 Phosphatidyl serine (Pu, DPu, FB-FY)
4 Phosphatidyl inositol (FPuR, DPu, FB-FY)
5 Phosphatidyl ethanolamine (Pi-Y, Y-Pu, Y-Pu)
6 Diphosphatidyl glycerol (Pu, FPu, Y)
7 Cerebroside sulfates (Pu, FPu, Y)
8 Cerebrosides (FY, Y, Y)
9 Lysophosphatidyl choline (FPi, FPu, FB)
10 Lysophosphatidyl serine (FPu, DPu, FB)
11 Lysophosphatidyl inositol (FPuR, DPu, FB)
12 Lysophosphatidyl ethanolamine (FPi, Pu, FB)
13 Phosphatidic acid (Pu, FPu, Y)
14 Free fatty acids (FB, DPu, B)
15 Nonpolar lipids (FY, Y, Y)
16 Gangliosides (Pu, FPu, Y)
17–19 Unidentified
20 Phosphatidyl glycerol ? (Pu, FPu, Y)
21–31 Unidentified

Colors are indicated by: Y, yellow; Pi, pink; Pu, purple; R, Red; B, blue; F, faint; and D, deep.

spots *13a* and *b*), whereas in others (dental pulp, cartilage, and bone) the spot was elongated. Phosphatidic acid synthesized from calf heart lecithin migrated as an elongated spot. The doublet effect could be due to different fatty acid composition or perhaps to ester and vinyl ether forms of the lipid. It is also possible that *13b* may be another unidentified phospholipid.

Many unidentified lipids were detected. Components designated *19*, *20*, and *21* were separated by silicic acid column chromatography in the same fraction as cardiolipin. On the basis of chromatograms of bacterial lipids, spot *20* is probably phosphatidyl glycerol. Components labeled *18*, *24*, *29*, and *30* were isolated from acidic extracts of preextracted, demineralized calcified tissues *18* and *24* being present as major constituents. Generally, however, most of the uncharacterized lipids were present as minor constituents of the tissue extracts. Nevertheless, components *20* and *21* were rapidly labeled (2 hr) after injection of ^{32}P and appeared to be as metabolically active as PA. By 13 hr both of these lipids, as well as PA, had lost most of their label.

Because of the number of unidentified lipids encountered in both neutral and acidic chloroform–methanol extracts of the tissues studied, the possibility of degradation artifacts naturally became a cause for concern. The involved preparative procedures required for calcified tissues very probably did give rise to certain artifacts, for sizeable amounts of lyso derivatives were found in the extracts of these tissues. On the other hand, little evidence of degradation resulting from the acidified chloroform–methanol extraction procedure was detected. When neutral chloroform–methanol extracts of rat liver were dried under vacuum, a residue of precipitated lipoprotein was obtained. This lipoprotein was reextracted with acidified chloroform–methanol to release the protein-bound lipids. Chromatography of the released lipids revealed no components different from those obtained from the neutral chloroform–methanol extracts from which the lipoprotein originated. Similarly, artifacts did not appear to arise during chromatography. When freshly prepared lipid extracts were chromatographed, chromatograms free from tailing or diffuse streaking were obtained. The comet-streaking of PS during chromatography in solvent 5 did not appear to be the result of degradation, but rather the result of a gradual change from the free acid to the salt form.

The determination of phosphorus described here is particularly well suited to chromatographic analysis. The residual silica gel from the digested paper spots is centrifuged down to the lower layer and does not interfere. In addition, the use of a two-stage method simplifies P analysis of lipid extracts in which a wide range in the amount of the various phospholipids occurs. The intensity of the yellow phosphomolybdic acid color in the upper phase serves as a useful guide in determining the size of aliquot needed for the final reduction to the blue phosphomolybdous acid.

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TABLE 2 Distribution of Polar Lipids in Extracts from Various Mammalian Tissues

No. on Fig. 3	Lipid	Percentage of Total Lipid Phosphorus				
		Rat		Calf	Fetal Calf	
		Brain	Liver	Heart	Cartilage	Bone
1	Sphingomyelin	5.7 ± 0.2	4.2 ± 0.2	9.1 ± 0.2	5.6 ± 0.1	—
2	Phosphatidyl choline	36.8 ± 1.3	50.8 ± 0.4	42.8 ± 0.5	19.0 ± 1.7	4.0 ± 0.3
3	Phosphatidyl serine	11.8 ± 0.8	3.2 ± 0.1	3.4 ± 0.1	8.6 ± 0.1	48.8 ± 0.3
4	Phosphatidyl inositol	3.1 ± 0.2	7.2 ± 0.2	3.4 ± 0.1	—	8.8 ± 0.4
5	Phosphatidyl ethanolamine	36.4 ± 1.0	25.2 ± 0.5	26.6 ± 0.2	13.6 ± 0.5	4.6 ± 0.4
6	Diphosphatidyl glycerol	2.2 ± 0.3	4.8 ± 0.1	12.1 ± 0.2	3.8 ± 0.9	3.2 ± 0 1
7	Cerebroside sulfates	(+)	—	—	—	(+)
8	Cerebrosides	(+)	—	—	—	—
9	Lysophosphatidyl choline	—	1.4 ± 0.2	0.4 ± 0.1	6.7 ± 0.1	1.7 ± 0.4
10	Lysophosphatidyl serine	—	0.2 ± 0.1	—	—	10.1 ± 0.4
11	Lysophosphatidyl inositol	—	0.4 ± 0.1	—	—	—
12	Lysophosphatidyl ethanolamine	—	—	—	14.1 ± 1.2	10.3 ± 0.2
13a	Phosphatidic acid	1.2 ± 0.2	0.4 ± 0.1	—	—	—
13b	Phosphatidic acid	1.1 ± 0.5	0.4 ± 0.2	—	—	—
14	Free fatty acids	—	—	(+)	—	(+)
15	Non-polar lipids	(+)	(+)	(+)	—	(+)
16	Gangliosides	(+)	—	—	—	—
17	Unidentified	—	—	—	5.4 ± 0.1	0.8 ± 0.2
18	"Chondrolipin"*	—	—	—	17.0 ± 1.6	2.7 ± 0.2
19	Unidentified	—	0.4 ± 0.2	—	—	1.0 ± 0.1
20	Phosphatidyl glycerol?	0.4 ± 0.2	0.5 ± 0.0	0.6 ± 0.1	2.2 ± 0.4	—
21	Unidentified	—	0.5 ± 0.1	0.5 ± 0.1	1.9 ± 0.7	1.2 ± 0.1
	Number of samples	4	4	4	2	2

Total lipid P analyzed for each sample was: rat brain, 5.36–9.70 μg; rat liver, 17.61–18.90 μg; calf heart, 9.14–9.53 μg; fetal calf proliferating cartilage, 3.82–3.93 μg; and fetal calf cancellous bone, 5.71–5.78 μg. Values are means ±sem. For nonphospholipids, (+) indicates detection on chromatograms. Nos. *22–26* on Fig. 3 are uncharacterized acidic polar lipids not containing P. No. *23* was found in extracts of bone marrow, spleen, lung, kidney, and liver. No. *24* was peculiar to calcified tissues and was the major component of extracts from the demineralized tissues. It has been labeled "calciolipin." Nos. *27–28* may be degradation artifacts from PE. Nos. *29–30* are ninhydrin-positive nonphospholipids found in acidified chloroform–methanol extracts of cartilage. No. *31* represented 1.4% of the lipid P in neutral chloroform–methanol extracts of demineralized cancellous bone.

* "Chondrolipin" was found almost exclusively in acidified chloroform–methanol extracts of cartilage, hence its name. It has been partially characterized and is distinct from any lipid previously described.

References

1. Stahl, E. *Pharmazie* **11:** 633, 1956.
2. Jatzkewitz, H. *Z. Physiol. Chem.* **320:** 134, 1960.
3. Wagner, H., L. Hörhammer, and P. Wolff. *Biochem. Z.* **334:** 175, 1961.
4. Müldner, H. G., J. R. Wherrett, and J. N. Cumings. *J. Neurochem.* **9:** 607, 1962.
5. Skipski, V. P., R. F. Peterson, and M. Barclay. *Biochem. J.* **90:** 374, 1964.
6. Lea, C. H., D. N. Rhodes, and R. D. Stoll. *Biochem. J.* **60:** 353, 1955.
7. Marinetti, G. V., and E. Stotz. *Biochim. Biophys. Acta* **21:** 168, 1956.
8. Marinetti, G. V. *J. Lipid Res.* **3:** 1, 1962.
9. Rouser, G., A. J. Bauman, N. Nicolaides, and D. Heller. *J. Am. Oil Chemists' Soc.* **38:** 565, 1961.
10. Hörhammer, L., H. Wagner, and G. Richter. *Biochem. Z.* **331:** 155, 1959.
11. Hörhammer, L., and G. Richter. *Biochem. Z.* **332:** 186, 1959.
12. Hörhammer, L., J. Hölzl, and H. Wagner. *Naturwiss.* **48:** 103, 1961 (abstract).
13. Skidmore, W. D., and C. Entenman. *J. Lipid Res.* **3:** 471, 1962.
14. Abramson, D., and M. Blecher. *J. Lipid Res.* **5:** 628, 1964.
15. Letters, R. *Biochem. J.* **93:** 313, 1964.
16. Marinetti, G. V. *J. Lipid Res.* **6:** 315, 1965.
17. Folch, J., M. Lees, and G. H. Sloane Stanley. *J. Biol. Chem.* **226:** 497, 1957.
18. Folch, J. In *Phosphorus Metabolism*, edited by W. D. McElroy and B. Glass. Johns Hopkins Press, Baltimore, 1952, Vol. 3, p. 186.
19. Wuthier, R. E. *J. Lipid Res.* **7:** 558, 1966.
20. Rouser, G., G. Kritchevsky, D. Heller, and E. Lieber. *J. Am. Oil Chemists' Soc.* **40:** 425, 1963.
21. Davidson, F. M., and C. Long. *Biochem. J.* **69:** 458, 1958.
22. Martin, J. B., and D. M. Doty. *Anal. Chem.* **21:** 965, 1949.
23. Dawson, R. M. C. *Biochem. J.* **75:** 45, 1960.
24. Dawson, R. M. C., N. Hemington, and J. B. Davenport. *Biochem. J.* **84:** 497, 1962.

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