Biochem. J. (1966) **100**, 354

A Two-Dimensional Thin-Layer Chromatographic Procedure for the Estimation of Plasmalogens

By K. OWENS
Department of Chemical Pathology, Institute of Neurology, National Hospital, Queen Square, London, W.C. 1

(*Received* 30 *December* 1965)

1. The use of two-dimensional thin-layer chromatography is described that allows the rapid and simultaneous determination of phospholipid classes and their constituent plasmalogens. 2. The method is based on the specific hydrolysis of plasmalogens to (2-acyl) lysophospholipid in the presence of a mercuric chloride spray reagent. 3. The proportion of mercuric chloride-labile phospholipid present in each phospholipid class, calculated on the basis of phosphorus recoveries from the charred chromatogram, was compared with the proportion of long-chain aldehyde and of total lipid phosphorus found in small-scale preparations of each class of phospholipid. 4. The method permits the determination of individual plasmalogens on preparations containing as little as 0·2 μg.atom of total lipid phosphorus.

The two-dimensional thin-layer chromatographic separation of lipid classes is now an established procedure when inadequate resolution of certain lipids has been obtained by unidimensional development (Skidmore & Entenman, 1962; Abramson & Blecher, 1964; Lepage, 1964; Rouser, Galli & Kritchevsky, 1965). However, since the slight structural difference between diacylphospholipids and analogous plasmalogens (1-alkenyl-2-acylphospholipids) militates against the chromatographic separation of the intact plasmalogens (Gray & Macfarlane, 1958; Long & Staples, 1961), existing methods for the estimation of plasmalogens are based on reactions that are specific for the $\alpha\beta$-unsaturated ether bond. Thus the lability of plasmalogens towards acid and their relative stability towards alkali compared with phospholipid ester linkages is the basis of methods involving the paper-chromatographic separation of the water-soluble hydrolysis products of phospholipids (Hack & Ferrans, 1959; Marinetti, Erbland & Stotz, 1959; Dawson, 1960; Dawson, Hemington & Davenport, 1962). Lability towards mild acid conditions, e.g. 90% (v/v) acetic acid (Marinetti *et al.* 1959), is also the basis for the determination of aldehydogenic lipid by the Schiff (fuchsin–sulphurous acid) reaction (Feulgen & Gruenberg, 1939; Anchel & Waelsch, 1944; Leupold & Büttner, 1953; Gray & Macfarlane, 1958; Sloane-Stanley & Bowler, 1962) and by the formation of aldehyde *p*-nitrophenylhydrazones (Wittenberg, Korey & Swenson, 1956; Rapport & Alonzo, 1960; Gottfried & Rapport, 1962). Alternatively, conditions have been described that permit the specific iodination of the $\alpha\beta$-unsaturated ether bond of plasmalogens (Siggia & Edsberg, 1948; Rapport & Franzl, 1957; Williams, Anderson & Jasik, 1962). Whereas the methods of plasmalogen estimation based on the quantitative chromatography of water-soluble hydrolysis products may be performed directly on tissue lipid extracts, other methods depend on the preliminary isolation of phospholipid classes, principally by column chromatography (e.g. Marinetti *et al.* 1959). The latter approach requires substantial quantities of lipid, and the possible contamination of phospholipid classes containing a small proportion of plasmalogen with a rich source of plasmalogen (frequently ethanolamine phospholipid) may incur considerable error. Again, the hydrolytic method of Dawson (1960) requires quantities of phospholipids that are sometimes unavailable (e.g. biopsy skeletal muscle). In addition, Rapport & Norton (1962) have noted that the hydrolytic method has been observed to provide low values for some plasmalogens when compared with results obtained by alternative methods, whereas procedures based on the Schiff reaction, on *p*-nitrophenylhydrazone formation and on specific iodination have occasionally produced equivocal plasmalogen values or required substantial correction factors (Rapport & Norton, 1962). The present method is based on the lability of plasmalogens towards mercuric chloride (Feulgen & Voit, 1924; Feulgen & Bersin, 1939; Norton, 1959; Schmidt *et al.* 1959; Hack & Ferrans, 1959; Müldner, Wherrett & Cumings, 1962; Norton &

Korey, 1962). The results have been compared with an estimate of aldehydogenic phospholipid obtained by the Schiff reaction.

Lipid classes, resolved by unidimensional thin-layer chromatography, are treated with a mercuric chloride spray reagent and the (2-acyl) lysophospholipids derived from plasmalogens separated from unaffected phospholipids (diacylphospholipids and glycerol ether phospholipids) by development in the second dimension. The total phosphorus contained in the charred lipid spots is then determined. The procedure permits the determination of individual plasmalogens present in smaller quantities of tissue lipid extracts than by existing methods.

MATERIALS AND METHODS

Preparation of lipid samples. The tissues used in the development of the method were the heart and whole brain of rabbit and mouse skeletal muscle. Rabbit heart and brain were weighed, homogenized in 19 vol. of analytical-grade $CHCl_3$–methanol (2:1, v/v) and filtered through sintered glass (porosity 3). These extracts were then washed with 0·2 vol. of 0·1 M-KCl according to the procedure of Folch, Lees & Sloane-Stanley (1957) and the lower phase was evaporated in a rotary evaporator. Lipid–protein bonds were split by evaporation (three times) from $CHCl_3$–methanol (2:1, v/v) containing 4% (v/v) of water (McIlwain & Rodnight, 1962) and residual water was removed as the $CHCl_3$ azeotrope. The residues were redissolved and made up to volume in $CHCl_3$–methanol (2:1, v/v).

For mouse skeletal muscle, the tissues were freed from visible fat and nerve, frozen in liquid N_2 and crushed to a pellet in a die. The pellet, which frequently weighed less than 0·5 g., was homogenized in 5 ml. of $CHCl_3$–methanol (2:1, v/v) and centrifuged at 1300*g* for 10 min. The supernatant was removed by teat pipette and the residue re-extracted (twice) in the same manner. The combined supernatants were then treated as described above.

Two dimensional thin-layer chromatography. Glass plates (20 cm. × 20 cm.) were spread with a film (nominal thickness 500 μ) of silica gel H (E. Merck A.-G., Darmstadt, Germany) slurried in ion-free water. Before activation at 110° for 30 min., these plates were washed with $CHCl_3$–methanol–water (60:35:8, by vol.), which removed to the top of the plate the bulk of an unidentified contaminant that charred strongly in the presence of H_2SO_4. Duplicate lipid samples, each containing 0·2–0·5 μg.atom of P, were added from a micrometer syringe (Burroughs, Wellcome and Co., London, N.W.1) as 1 cm. bands, 2 cm. in from the left and 3 cm. in from the right-hand edge, under a stream of N_2. The plate was developed in freshly prepared $CHCl_3$–methanol–water–acetic acid (65:43:3:1, by vol.), which is a modification of that described by Skipski, Peterson, Sanders & Barclay (1963). The chromatography tanks were lined with paper and were equilibrated for 1 hr. before use at ambient temperatures in the region of 20°. The development was discontinued when the solvent front was 4 cm. from the top of the plate. Volatile solvents were removed under a stream of cold air (approx. 10 sec.) and the chromatogram was redeveloped to the top of the plate with light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (80:20:1, by vol.). This solvent mixture caused the migration of lipids other than phospholipids and glycolipids to positions above the first solvent front. Residual acetic acid derived from the developing solvents was neutralized by supporting the chromatogram above aq. NH_3 (sp.gr. 0·880), contained in a sealed dish, for 5 min. The bulk of the excess of NH_3 was removed by evacuation ($\leqslant$0·5 mm. Hg) for 30 min. in a 10 in.-diam. desiccator (Montcrief, Perth). The left-hand lipid track was then sprayed with 5 mM-$HgCl_2$ (analytical grade) in ion-free water while the remainder of the chromatogram was screened. During a period of 1 min., the plate was turned through an angle of 90° and the position for the second-dimension solvent front (approx. 5 cm. from the top of the plate) was scored in the silica gel. The chromatogram was then reactivated by evacuation ($\leqslant$0·5 mm. Hg) over dark-blue self-indicating silica gel for 1 hr. The $HgCl_2$-treated lipids were then developed in the second dimension with $CHCl_3$–methanol–water (60:35:8, by vol.). The chromatogram was allowed to dry, sprayed with 18 N-H_2SO_4 and charred at 180° for 1 hr. in an oven situated beneath an extractor fan for the removal of volatilized H_2SO_4 and $HgCl_2$. Additional two-dimensional chromatograms could be run by starting at hourly intervals.

Charred areas were removed from the plate with a single-edged razor blade into 10 ml. stoppered tubes. Appropriate blank areas were also taken for each lipid spot, and blank areas of silica gel corresponding to large, medium and small-sized lipid spots were added to tubes containing evaporated samples (0·1 ml.) of a standard phosphate solution equivalent to 0·05 μg.atom of P. Total phosphorus was determined by the ultraviolet method of Sloane-Stanley & Eldin (1962). However, where a spot or corresponding blank area was of a size such that the silicic acid would absorb all the ashing mixture (0·15 ml.), the scrapings were apportioned to two tubes. The absence of free ashing mixture occasionally resulted in erroneous phosphorus values. The volume of ashing mixture could not be increased without maintaining the critical acid concentration by the addition of larger volumes of the other reagents, with a resulting decrease in sensitivity. Plasmalogen percentages were based on the quantities of (2-acyl) lysophospholipid phosphorus.

Preparative thin-layer chromatography of lipid classes. For this work, methanol and ethanol (both of analytical grade) were refluxed over KOH and zinc and distilled. A sample (0·2 ml.) of a rabbit tissue lipid extract, containing not more than 7 μg.atoms of P, was added to a washed activated plate of silica gel H (Merck) as a band (15 cm.) and developed in $CHCl_3$–methanol–water–acetic acid (65:43:3:1, by vol.) up to a scored line 4 cm. from the top of the plate. The chromatogram was then fumed with NH_3 for 5 min. and the excess of NH_3 removed under high vacuum as described above. The entire plate was immediately and lightly sprayed with a 0·2% (w/v) solution of dichlorofluorescein R (Hopkin and Williams Ltd., Chadwell Heath, Essex) in aq. 50% (v/v) ethanol and the positions of the lipid bands were quickly marked when viewed in ultraviolet light. The chromatogram was covered with a glass plate and rectangles of measured height containing the lipid bands were successively scraped off into tubes containing appropriate solvents [i.e. $CHCl_3$–methanol (1:1, v/v) for all fractions except the band running with the solvent front, which was placed into $CHCl_3$ only]. The

silica slurries were transferred by wide-jet teat pipettes to chromatography columns (0·5 cm. × 10 cm.) each fitted with a sintered-glass disk (porosity 2). For the lipids present in the solvent-front band of the preparative thin-layer chromatogram, neutral lipids were eluted with $CHCl_3$ before the elution of polyglycerophospholipid with 25 ml. of $CHCl_3$–methanol (2:1, v/v). The other columns were eluted with 25 ml. of $CHCl_3$–methanol (1:1, v/v). The eluates were evaporated to dryness in a rotary evaporator at 35°, and the residues were redissolved in $CHCl_3$–methanol (2:1, v/v) and quantitatively transferred from the flasks to graduated centrifuge tubes, made up to a suitable volume (usually 2 ml.) and assayed for total phosphorus. Thin-layer plates to which tissue lipid had not been applied were processed in the same manner, rectangles equal in area to those occupied by lipid bands being removed to provide blank controls for the phosphorus-containing column eluates.

Estimation of aldehydogenic lipid. Samples from each phospholipid preparation were assayed for aldehydogenic lipid by the Schiff procedure of Sloane-Stanley & Bowler (1962), with palmitaldehyde dimethylacetal as the standard. The lipid samples were hydrolysed in the presence of 0·20 ml. of acetic acid containing 0·02 ml. of 1·5 mM-$HgCl_2$ in 0·5 N-HCl. This reagent was described by Pietruszko & Gray (1962) for the additional hydrolysis of adventitious cyclic acetals.

RESULTS

Lysophospholipids produced by residual acetic acid on the plates. In Table 1 a comparison is made between the average percentages of choline and ethanolamine lysophospholipids produced during the two-dimensional thin-layer chromatography of mouse skeletal-muscle total lipid extracts when alternative trial procedures are followed. When the residual acetic acid derived from the first-dimensional solvent mixtures was not neutralized in an ammonia-containing atmosphere, an unspecific hydrolysis of plasmalogens and diester phospholipid occurred (procedure 2), usually accompanied by streaking of the second-dimensional spots. These undesirable effects could be eliminated by treatment with ammonia (procedure 4). The specific hydrolysis of plasmalogens by mercuric chloride (procedure 3) was in basic agreement with average percentages of aldehydogenic choline and ethanolamine phospholipids (2 and 28% respectively) as determined by the Schiff reaction on thin-layer chromatographic preparations of mouse skeletal-muscle choline- and ethanolamine-containing phospholipids. However, the small quantity of choline plasmalogen in this tissue made exact comparison difficult.

Rabbit-heart choline- and ethanolamine-containing phospholipid preparations (35 and 51% of aldehydogenic phospholipid respectively) were subjected to unidimensional thin-layer chromatography with the two acetic acid-containing solvent mixtures. No decomposition products such as lysophospholipids or cyclic acetals were observed, each preparation running as a single spot.

The mercuric chloride spray reagent had no effect on diester phospholipid during two-dimensional thin-layer chromatography in which the plate was treated with ammonia. With a sample of ovolecithin (given by Professor C. Long) the same quantity of phosphorus was recovered from the single second-dimensional spot as from a duplicate sample run in the first dimension only. Tattrie (1959) reported the presence of only acyl groups in ovolecithin and the present sample gave no Schiff reaction.

Applications of the method to rabbit-heart and -brain phospholipids. The diagrammatic appearance of a typical charred two-dimensional mercuric chloride-treated chromatogram of a rabbit-heart lipid extract (Fig. 1) may be compared with one obtained when the left-hand lipid track had been sprayed with ion-free water (Fig. 2). When a rabbit-brain lipid extract was resolved by two-dimensional mercuric chloride thin-layer chromatography, two very prominent spots appeared in the first dimension, between phosphatidylethanolamine and cardiolipin (polyglycerophospholipid), and were resolved into three components in the second

Table 1. *Extent of the hydrolytic formation of choline and ethanolamine lysophospholipids during two-dimensional thin-layer chromatography when different procedures are used*

Results were obtained with total lipid extracts of the hind-leg muscle from two mice. Numbers in parentheses denote the numbers of determinations providing the average percentages.

Procedure	Spray reagent	Treatment with NH_3	$\frac{\text{Lysophospholipid P}}{\text{Total phospholipid P}} \times 100$ Choline	Ethanolamine
1	$HgCl_2$ (5 mM)	−	14 (2)	38 (2)
2	Ion-free water	−	10 (2)	11 (3)
3	$HgCl_2$ (5 mM)	+	3 (3)	29 (3)
4	Ion-free water	+	0 (2)	0 (2)

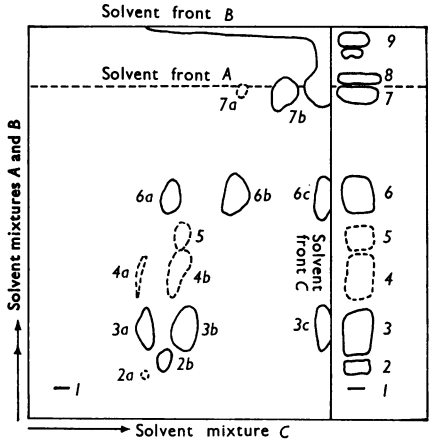

Fig. 1. Two-dimensional thin-layer chromatogram of a lipid extract from the heart of an adult rabbit (65 μl. samples contained 0·497 μg.atom of P). The left-hand lipid track was sprayed with 5 mM-$HgCl_2$ before second-dimensional development. Identity of solvent mixtures: *A*, $CHCl_3$–methanol–water–acetic acid (65:43:3:1, by vol.); *B*, light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (80:20:1, by vol.); *C*, $CHCl_3$–methanol–water (60:35:8, by vol.). Identity of spots, with parent phospholipid in parentheses: *1*, origins; *2*, sphingomyelin plus native lysolecithin; *2a*, native lysolecithin; *2b*, sphingomyelin; *3*, choline phospholipids; *3a*, (2-acyl) lysophosphatidylcholine (choline plasmalogen); *3b*, phosphatidylcholine; *3c* and *6c*, β-chloromercurialdehyde (tentative identification); *4*, serine phospholipids; *4a*, (2-acyl) lysophosphatidylserine (serine plasmalogen); *4b*, phosphatidylserine; *5*, phosphatidylinositol; *6*, ethanolamine phospholipids; *6a*, (2-acyl) lysophosphatidylethanolamine (ethanolamine plasmalogen); *6b*, phosphatidylethanolamine; *7*, impure cardiolipin; *7a*, unidentified phospholipid; *7b*, cardiolipin; *8*, cholesterol; *9*, other neutral lipids.

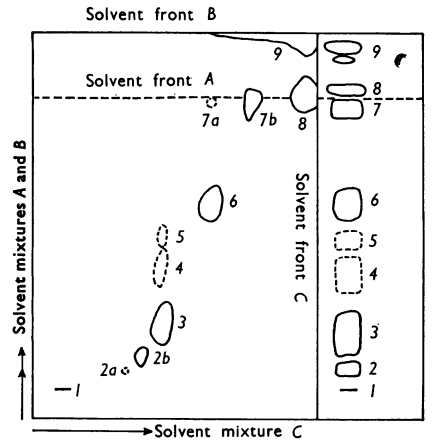

Fig. 2. Two-dimensional thin-layer chromatogram of a lipid extract from a heart of an adult rabbit (50 μl. samples contained 0·382 μg.atom of P). The left-hand lipid track was sprayed with ion-free water before second-dimensional development. Identities of solvent mixtures and spots are as given in Fig. 1.

dimension. These spots (together with the cholesterol spots) turned purple during the initial stages of charring with sulphuric acid and were tentatively designated as glycolipids. Faint spots in similar positions were occasionally observed with the heart extracts but are not shown in Figs. 1 and 2. Native lysolecithin was only resolved from sphingomyelin by second-dimensional development.

Uncharred two-dimensional mercuric chloride-treated chromatograms of rabbit-heart lipid were sprayed with a colourless mercury-sensitive reagent consisting of equal volumes of 2,2′-bipyridyl (1%, w/v, in ethanol), aq. 1% (w/v) potassium ferrocyanide solution and 2N-ammonia, as a modification of the spot-test conditions described by Feigl (1960). The two second-dimensional spots, tentatively identified as long-chain aldehydes derived from choline and ethanolamine plasmalogens, were stained red. Norton (1959) has described the formation of β-chloromercurialdehydes after the treatment of plasmalogens with mercuric chloride on filter paper.

The two-dimensional chromatographic analysis of seven rabbit hearts and one rabbit brain in terms of individual phospholipid phosphorus as percentages of the total phospholipid phosphorus is shown in Table 2. Phosphorus was not recovered in significant quantities from spots above cardiolipin. The values found for the immature rabbits would have been included with the average percentages of phospholipids obtained for the five hearts from mature animals had it not been for the relatively low percentages obtained for choline plasmalogen in the hearts of 6-week-old and 2-day-old animals. The percentages of ethanolamine plasmalogen for these hearts from young rabbits were just below the range of values found for the hearts from adult animals. It was not decided whether the relatively low plasmalogen content was attributable to immaturity or to biological variation.

Comparison of the mercuric chloride method with the Schiff method. Each tissue lipid extract (except the lipid extract of the heart of a 2-day-old animal) was subjected to preparative thin-layer chromatography involving a total of approx. 7 μg.atoms of phospholipid P, and the constituent sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, cardiolipin and neutral lipid were recovered from the

Table 2. *Distribution of individual phospholipids in rabbit heart and whole brain, as determined by two-dimensional thin-layer chromatography with mercuric chloride spray reagent for the hydrolysis of plasmalogens*

Results express the phosphorus of individual phospholipids as percentages of the total phospholipid phosphorus applied to the plate. Numbers in parentheses denote the ranges of results.

	Heart (five adults)	Heart (6-week-old)	Heart (2-day-old)	Brain (whole)
Total phospholipid (μg.atoms of P/g. of wet tissue)	26·7 (23·3–29·5)	29·4	20·4	75·7
Native lysolecithin	0·7 (0·4–1·2)	1·2	—	0·4
Sphingomyelin	5·0 (3·8–5·7)	4·9	6·2	12·4
Choline phospholipid*	42·2 (39·2–43·5)	42·3	41·8	32·2
Choline plasmalogen	15·1 (11·8–18·0)	6·6	10·3	1·0
Serine phospholipid*	3·3 (2·9–3·6)	2·2	3·9	15·8
Serine plasmalogen	0·7 (0·1–1·4)	—	—	1·0
Monophosphoinositide	3·6 (3·3–4·2)	3·5	5·0	3·0
Ethanolamine phospholipid*	30·9 (29·5–32·0)	33·5	28·9	35·3
Ethanolamine plasmalogen	15·4 (14·7–15·9)	12·8	12·3	22·6
Cardiolipin	11·6 (9·7–13·2)	11·7	10·7	2·0
Unidentified phospholipid	1·0 (0·5–1·4)	0·7	0	—
Recovery (average percentage of total lipid P analysed)	98·3	100·0	96·4	101·1

* Total phospholipid class, i.e. diacylphospholipid, plasmalogen and glycerol ether phospholipid (if any).

chromatographic bands as described above. These preparations were then analysed for plasmalogen aldehyde by the Schiff reaction and for total phosphorus. It was found unnecessary to remove the dichlorofluorescein from these preparations since the extinction of this substance was very small at the wavelength used for the estimation of plasmalogen aldehyde (551 mμ) and dichlorofluorescein was destroyed by the ashing procedure in the determination of total phosphorus. Dichlorofluorescein could, however, be effectively removed from phospholipids dissolved in chloroform–methanol (2:1, v/v) by washing with 0·2 vol. of 0·1 M-potassium chloride (Folch *et al.* 1957) and washing (three times) the resulting lower phase with synthetic upper phase [chloroform–methanol–0·1 M-potassium chloride (2:20:19, by vol.)].

The antioxidant butylated hydroxytoluene could not be added to the solvent mixtures (5 mg./100 ml.) used in the preparation of these lipid samples since it severely decreased their response towards the Schiff reagent (K. Owens, unpublished work). However, this substance did not affect the quantity of mercuric chloride-labile phospholipid produced during two-dimensional thin-layer chromatography when present in the developing solvents at the same concentration.

In the derivation of the percentages of aldehydogenic phospholipids shown in Table 3, the phosphorus content of each phospholipid preparation was corrected for the phosphorus recovered from the column eluate blank controls (0·0095 $\pm$ 0·0005 μg.atom of P, average of five experiments). The values were compared with the corresponding percentages of mercuric chloride-labile phospholipid phosphorus found for each phospholipid class by two-dimensional mercuric chloride thin-layer chromatography. The preparations of sphingomyelin, phosphatidylinositol, cardiolipin and the neutral-lipid fraction from five hearts from adult rabbits produced insignificant responses towards the Schiff reagent, and chromatographic spots attributable to the mercuric chloride-lability of these lipids were not observed. For the total lipid extracts (Table 3, column 1) the mercuric chloride-labile phospholipid percentages were based on the total phosphorus recovered from the (2-acyl) lysophospholipid spots produced by the action of mercuric chloride on the choline-, serine- and ethanolamine-containing phospholipids, and the total phospholipid phosphorus applied to the plate. The degree of correspondence between the value for the total mercuric chloride-labile phosphorus and of direct estimates of aldehydogenic phospholipid made on the tissue lipid extracts (Table 3, column 1) emphasized previous indications that no plasmalogen hydrolysis had occurred in the first-dimensional development, and that the mercuric chloride had produced complete plasmalogen hydrolysis with respect to estimates of total Schiff-positive phospholipid. Since the actual quantities of mercuric chloride-labile phosphorus and plasmalogen aldehyde attributed to rabbit-

Table 3. *Comparison between the percentages of mercuric chloride-labile and aldehydogenic phospholipids of rabbit heart and brain*

Results express the percentages of those phospholipid classes observed to exhibit lability towards the $HgCl_2$ spray reagent during two-dimensional thin-layer chromatography, and of aldehydogenic phospholipid estimated to be present in preparations of each phospholipid class by the Schiff reaction. Numbers in parentheses denote the numbers of determinations providing the average percentages.

		Total lipid extract		Choline phospholipid		Ethanolamine phospholipid		Serine phospholipid	
Rabbit	Tissue	$HgCl_2$-labile	Schiff-positive	$HgCl_2$-labile	Schiff-positive	$HgCl_2$-labile	Schiff-positive	$HgCl_2$-labile	Schiff-positive
1	Heart (adult)	33 (1)	36 (1)	39 (1)	40 (1)	50 (2)	48 (1)	6 (2)	2 (1)
2	Heart (adult)	32 (3)	33 (4)	31 (3)	27 (4)	50 (2)	49 (4)	—	9 (2)
3	Heart (adult)	27 (1)	27 (1)	29 (3)	27 (1)	47 (2)	47 (1)	7 (2)	11 (1)
4	Heart (adult)	29 (1)	29 (1)	35 (2)	35 (2)	49 (2)	51 (2)	4 (1)	9 (1)
5	Heart (adult)	34 (1)	35 (2)	42 (1)	39 (1)	49 (2)	47 (1)	—	12 (1)
6	Heart (6-week-old)	20 (2)	21 (2)	16 (2)	12 (1)	39 (1)	45 (1)	—	—
5	Brain (whole)	25 (1)	26 (1)	3 (1)	2 (1)	66 (2)	66 (1)	3 (1)	3 (1)

Table 4. *Comparison between the percentages of mercuric chloride-labile choline and ethanolamine phospholipids estimated to be present in rabbit-heart and -brain total lipid extracts, and in preparations of these phospholipids, by using two-dimensional thin-layer chromatography*

Numbers in parentheses denote the number of determinations providing the average percentages.

		Choline phospholipid		Ethanolamine phospholipid	
Rabbit	Tissue	Total lipid extract	Preparation	Total lipid extract	Preparation
4	Heart (adult)	35 (2)	31 (1)	49 (2)	50 (1)
5	Heart (adult)	42 (1)	40 (2)	49 (2)	47 (2)
6	Heart (6-week-old)	16 (2)	10 (1)	39 (1)	42 (1)
5	Brain (whole)	3 (1)	—	66 (2)	62 (1)

heart and -brain phosphatidylserine were relatively small compared with the quantities of choline and ethanolamine plasmalogens present, there were considerable differences between the corresponding percentages (Table 3, column 4) and no statistical comparison of the results obtained by the two methods was attempted. However, for the total lipid extracts and the choline and ethanolamine phospholipids, a statistical comparison between the percentages of plasmalogen, as estimated by the two methods, provided a sample correlation coefficient of 0·987 for the 21 pairs of plasmalogen percentages (Table 3, columns 1–3). The critical absolute value of correlation coefficient at the 1% level of significance was 0·549. When the correlation coefficients for the seven pairs of percentages obtained for choline and ethanolamine plasmalogens were taken separately, values of 0·991 and 0·938 were obtained respectively. The corresponding critical absolute value of correlation coefficient at the 1% level of significance (0·874) was again exceeded. It was therefore inferred that the mercuric chloride method was equally responsive (with respect to the Schiff reaction) towards choline and ethanolamine plasmalogens.

The procedure involving the preparative thin-layer chromatography and subsequent recovery of phospholipid classes might conceivably have incurred a substantial decrease in the proportion of Schiff-positive phospholipids and hence the fortuitous agreement of values shown in Table 3 (columns 2 and 3). The percentages of mercuric chloride-labile phospholipid phosphorus present in the preparations of choline and ethanolamine phospholipids from rabbits 4, 5 and 6 were therefore determined. A comparison between these results and the corresponding percentages of mercuric chloride-labile choline and ethanolamine phospholipids, as determined directly on the tissue lipid extracts (Table 3), is shown in Table 4. The differences, calculated to be an average fall of 3%, presumably represented the average loss of plasmalogen incurred in the preparation of phospholipid samples.

DISCUSSION

The exact location of cardiolipin on the charred chromatogram in both the first and second dimensions is only revealed when the neutral lipids are removed by redevelopment with the light petroleum–diethyl ether–acetic acid solvent mixture. If this is not done, then the large charred area that must be removed from the plate for the estimation of cardiolipin phosphorus is excessive and creates difficulties in the ashing procedure, as described above. If the estimation of cardiolipin and the minor unidentified phospholipid having the same first-dimensional mobility are not required, this redevelopment may be omitted.

With regard to choline, serine and ethanolamine lysophospholipid second-dimensional spots observed when chromatograms were not fumed with ammonia and sprayed with ion-free water in place of mercuric chloride solution, the results may be compared with the report of Abramson & Blecher (1964). These authors performed the two-dimensional thin-layer chromatography of phospholipids isolated from rat thymus with the first-dimensional solvent mixture chloroform–methanol–acetic acid–water (250:74:19:3, by vol.) and then dried the chromatogram in air for 15min. before development in the second dimension. The appearance of lysophospholipids was not reported and it might be inferred that the use of 10mM-sodium carbonate in the preparation of the silica gel G (Merck) thin-layer plates prevented the acetic acid hydrolysis of phospholipids. Alternatively, the relatively mild charring technique used (10N-sulphuric acid heated at 110° for 15min.) has proved inadequate in the present author's experience for the elucidation of minor phospholipids. The partial hydrolysis of phospholipids during chromatography on silicic acid-impregnated paper with acetic acid-containing solvent mixtures has been limited by the use of reduced ambient temperatures (Hack & Ferrans, 1959; Marinetti *et al.* 1959; Kates & James, 1961). In the present method, thin-layer chromatography was carried out at ambient temperatures in the region of 20°. When chromatograms were fumed with ammonia and the left-hand lipid track was sprayed with ion-free water, the absence of lysophospholipid spots was in agreement with the report of Thiele (1959), who found no liberation of aldehyde when purified phospholipid emulsions, free from heavy-metal catalysts, were incubated for several hours at 37°. In this context, the complete liberation of plasmalogen aldehyde by mercuric chloride (with respect to results obtained by the Schiff method) does not require the presence of acid, assuming that the silicic acid had been neutralized by the ammonia treatment. This is in accord with the report of Schmidt *et al.* (1959), who obtained quantitative plasmalogen hydrolysis of brain kephalin by emulsification in aqueous mercuric chloride without the addition of acid. The catalytic hydrolysis of plasmalogens by aqueous mercuric chloride may be especially effective during thin-layer chromatography since the phospholipids are adsorbed on to a finely divided hygroscopic support in intimate contact with the aqueous phase.

The basic agreement between the chromatographic and Schiff-reaction methods (Table 3) was not attributable to the relatively dilute mercuric chloride included in the hydrolysis mixture for the Schiff method, since the plasmalogen percentages obtained by hydrolysing the phospholipid samples in acetic acid (0·20ml.) containing ion-free water (0·02ml.) were only 1–2% lower (K. Owens, unpublished work).

Rapport & Lerner (1959), using methods involving the formation of *p*-nitrophenylhydrazones and the uptake of iodine for the estimation of total plasmalogen in rabbit heart and whole brain, reported values (corrected by Rapport & Norton, 1962) of 32 and 27% respectively. These compare favourably with average total plasmalogen values of 31 and 32% for heart from adult rabbits obtained here by the mercuric chloride and Schiff methods respectively, whereas the single preparation of rabbit-brain lipid afforded values of 25 and 26% as total plasmalogen by these two methods.

As a possible extension to the mercuric chloride method, it may be feasible to estimate individual plasmalogen aldehydes by their recovery from the second-dimensional spots tentatively identified as β-chloromercurialdehyde (Norton, 1959), followed by their quantitative gas–liquid chromatography as dimethylacetals.

This work was supported by the Muscular Dystrophy Group of Great Britain. The author is indebted to Dr G. H. Sloane-Stanley for the details of the total phosphorus and Schiff reaction methods, and thanks Professor C. Long for the sample of ovolecithin, Mrs F. F. Frais for the chromatographic analysis of the heart from 2-day-old rabbit and Dr B. P. Hughes and Mr T. Skrbic for their interest.

REFERENCES

Abramson, D. & Blecher, M. (1964). *J. Lipid Res.* **5**, 628.
Anchel, M. & Waelsch, H. (1944). *J. biol. Chem.* **152**, 501.
Dawson, R. M. C. (1960). *Biochem. J.* **75**, 45.
Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962). *Biochem. J.* **84**, 497.
Feigl, F. (1960). *Spot Tests in Organic Analysis*, p. 108. London: Elsevier Publishing Co.
Feulgen, R. & Bersin, T. (1939). *Hoppe-Seyl. Z.* **260**, 217.
Feulgen, R. & Gruenberg, H. (1939). *Hoppe-Seyl. Z.* **257**, 161.
Feulgen, R. & Voit, K. (1924). *Pflüg. Arch. ges. Physiol.* **206**, 389.

Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.

Gottfried, E. L. & Rapport, M. M. (1962). *J. biol. Chem.* **237**, 329.

Gray, G. M. & Macfarlane, M. G. (1958). *Biochem. J.* **70**, 409.

Hack, M. H. & Ferrans, V. J. (1959). *Hoppe-Seyl. Z.* **315**, 157.

Kates, M. & James, A. T. (1961). *Biochim. biophys. Acta*, **50**, 478.

Lepage, M. (1964). *J. Chromat.* **13**, 99.

Leupold, F. & Büttner, H. (1953). *Hoppe-Seyl. Z.* **292**, 13.

Long, C. & Staples, D. A. (1961). *Biochem. J.* **80**, 557.

McIlwain, H. & Rodnight, A. (1962). *Practical Neurochemistry*, p. 64. London: J. and A. Churchill Ltd.

Marinetti, G. V., Erbland, J. & Stotz, E. (1959). *Biochim. biophys. Acta*, **31**, 251.

Müldner, H. G., Wherrett, J. R. & Cumings, J. N. (1962). *J. Neurochem.* **1**, 607.

Norton, W. T. (1959). *Nature, Lond.*, **184**, 1144.

Norton, W. T. & Korey, S. R. (1962). *Proc. 4th int. Congr. Neuropathology*, p. 186. Stuttgart: George Thieme Verlag.

Pietruszko, R. & Gray, G. M. (1962). *Biochim. biophys. Acta*, **56**, 232.

Rapport, M. M. & Alonzo, N. (1960). *J. biol. Chem.* **235**, 1953.

Rapport, M. M. & Franzl, R. E. (1957). *J. Neurochem.* **1**, 303.

Rapport, M. M. & Lerner, B. (1959). *Biochim. biophys. Acta*, **33**, 319.

Rapport, M. M. & Norton, W. T. (1962). *Annu. Rev. Biochem.* **31**, 103.

Rouser, G., Galli, C. & Kritchevsky, G. (1965). *J. Amer. Oil Chem. Soc.* **42**, 404.

Schmidt, G., Ottenstein, B., Spencer, W. A., Keck, K., Blietz, R., Papas, J., Porter, D., Levin, M. L. & Thannhauser, S. L. (1959). *Amer. J. Dis. Child.* **97**, 691.

Siggia, S. & Edsberg, R. L. (1948). *Analyt. Chem.* **20**, 762.

Skidmore, W. D. & Entenman, C. (1962). *J. Lipid Res.* **3**, 471.

Skipski, V. P., Peterson, R. F., Sanders, J. & Barclay, M. (1963). *J. Lipid Res.* **4**, 227.

Sloane-Stanley, G. H. & Bowler, L. M. (1962). *Biochem. J.* **85**, 34P.

Sloane-Stanley, G. H. & Eldin, A. K. (1962). *Biochem. J.* **85**, 40P.

Tattrie, N. H. (1959). *J. Lipid Res.* **1**, 60.

Thiele, O. W. (1959). *Hoppe-Seyl. Z.* **316**, 137.

Williams, J. N., Anderson, C. E. & Jasik, A. D. (1962). *J. Lipid Res.* **3**, 378.

Wittenberg, J. B., Korey, S. R. & Swenson, F. H. (1956). *J. biol. Chem.* **219**, 39.