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A Hydrolytic Procedure for the Identification and Estimation of Individual Phospholipids in Biological Samples

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(*Received* 21 *August* 1959)

Procedures which have been developed for the assay of individual phospholipids within a mixed-lipid sample isolated from tissues, usually depend upon a total hydrolysis of the phospholipids followed by separate determinations of the hydrolysis products, e.g. choline, phosphorus, inositol, serine, ethanolamine and sphingosine. Not only does this require the setting up of a large number of individual time-consuming assays, but the method suffers from the disadvantage that it fails to distinguish between the plasmalogens and the usual di-acylated phospholipids, e.g. between choline plasmalogen and lecithin.

The present method is based on one developed some years ago for the examination of phospholipid fractions by paper chromatography after mild alkaline hydrolysis (Dawson, 1954*a*). Such a hydrolysis results in the removal of the fatty acids from the alkali-labile phospholipids, leaving recognizable water-soluble fragments (mainly the corresponding esters of glycerophosphoric acid, e.g. glycerylphosphorylcholine) which can be subsequently separated and estimated by paper chromatography. This procedure has been simplified so that quantitative recoveries of the deacylated phospholipid fragments can be achieved, and extended so that the alkali-stable phospholipids (defined in this paper as the phosphorus remaining soluble in lipid solvent after alkaline hydrolysis) can also be examined. Thus the alkali-stable phospholipids are subjected to mild acid hydrolysis when the deacylated plasmalogens break down, giving a long-chain aldehyde and their corresponding water-soluble phosphodiesters. Finally the residual phospholipids are hydrolysed in methanolic hydrochloric acid, when sphingomyelin gives phosphorylcholine and sphingosylphosphorylcholine (Rennkamp, 1949; Dawson, 1958*a*).

The method requires the use of only a single assay procedure (for phosphorus) and it is possible to resolve and assay complex lipid samples within 2 days.

EXPERIMENTAL

Preparation of lipid samples

The procedure of Folch, Lees & Sloane-Stanley (1957) was mainly followed. The tissue samples were homogenized in 24 vol. of $CHCl_3$–methanol (2:1, v/v) in a high-speed blender. After standing for 30 min. with stirring, the suspension was centrifuged and filtered. The filtrate was shaken for 2–3 min. with 0·2 vol. of 0·9% NaCl soln. After centrifuging the emulsion, or leaving it overnight at 0°, the upper aqueous layer containing any water-soluble phosphorus impurities was removed and discarded. The lower layer was taken to dryness *in vacuo*, dissolved in an equal volume of $CHCl_3$, analysed for total phosphorus and stored at −20° until required.

As the subsequent alkaline hydrolysis of the phospholipids was performed with a limited amount of alkali, the method was not directly applicable to lipid extracts containing large amounts of neutral fat and low concentrations of phospholipid (< 40%, w/w), e.g. extracts of adipose tissue or fatty livers. With such extracts it is first necessary to separate the phospholipids with a simple silicic acid column or by other means (Borgström, 1952; Barron & Hanahan, 1958).

Alkaline hydrolysis of the phospholipids

Method I. The lipid sample, containing about 500 μg. of lipid P (not more than 550 μg.) was pipetted into a 50 ml. round-bottomed flask with a ground-glass joint fitted with a standard splash head. The solvent was removed at 40–50° *in vacuo* with constant swirling to avoid splashing. The residue was redissolved in 0·8 ml. of A.R. carbon tetrachloride (ethanol, methanol or light petroleum can also be used). It was found essential to use pure carbon tetrachloride as some technical grades gave pink spots on the subsequently developed chromatograms. Ethanol (7·5 ml.) was then added, followed by 0·65 ml. of water and 0·25 ml. of aq. N-NaOH. The mixture was incubated at 37° for 20 min. and the pH tested with indicator paper. The hydrolysate was still distinctly alkaline, provided that the lipid sample used did not contain more than 550 μg. of lipid P or excess of esterified neutral fat. Ethyl formate (0·4 ml.) was then added and, after mixing, the solution was incubated for a further 5 min. at 37°. This neutralized excess of alkali by the reaction: ethyl formate + NaOH = sodium formate + ethanol. The hydrolysate was then taken to dryness *in vacuo* below 60°. A volume of water was equilibrated with 2 vol. of *iso*butanol–$CHCl_3$ mixture (1:2, v/v), by shaking for a few minutes and allowing to settle. Samples from the upper aqueous layer (1 ml.) and lower solvent layer (2 ml.) were added to the dry residue of the hydrolysate, and the flask was shaken and warmed slightly to ensure the quantitative solubilization of the hydrolysis products and the alkali-stable phospholipids. The emulsion was then transferred as completely as possible to a centrifuge tube. After centrifuging, the upper aqueous layer (A) was withdrawn with a Pasteur pipette and stored for chromatography at 0°.

Method II. The hydrolysis was conducted in the same way as in method I. However, alkali was removed by passing the hydrolysate cooled to 0° through a short column of Amberlite IRC 50 H^+ (3 cm. × 0·8 cm. bore). The hydrolysis products were washed through the column with two successive 5 ml. portions of 80% ethanol. The total effluent of the column was taken to dryness *in vacuo* and then distributed between water and *iso*butanol–$CHCl_3$ as in method I. The water-soluble phosphorus-containing hydrolysis products passed quantitatively through the resin column but the alkali-stable phospholipids were partially adsorbed. For this reason the resin columns were never regenerated and method II could not be used for the subsequent examination of the alkali-stable phospholipids.

Hydrolysis of the plasmalogens

From the slightly turbid lower solvent layer containing the alkali-stable phospholipids, a sample (1·6 ml.) was withdrawn with a pipette operated by a hand-controlled suction bulb. To ensure that this fraction was not contaminated with any of the aqueous layer, the point of the pipette was inserted below the interface and any entrapped water driven out by slightly warming the pipette with the hand. To the sample contained in a 10 ml. stoppered centrifuge tube was added 0·8 ml. of 10% (w/v) trichloroacetic acid solution. The mixture was vigorously shaken (mechanically) for 30 min. in a water bath at 37°. On cooling, 2 ml. of light petroleum (b.p. 40–60°) was added and, after shaking, the tube was centrifuged. The upper solvent layer containing the alkali- and acid-stable phospholipids was removed as completely as possible with a Pasteur pipette. The lower aqueous layer containing the hydrolysed plasmalogen phosphorus was washed twice in the centrifuge with 5 ml. of $CHCl_3$–ether (1:4, v/v). This removed the remaining alkali- and acid-stable phospholipids and also the trichloroacetic acid. The aqueous layer was then adjusted to a neutral or slightly alkaline pH by holding a wick soaked in dilute ammonia solution in the air of the tube for a few seconds, which was then stoppered and shaken. It was stored at 0° for chromatography (B).

Hydrolysis of phospholipids stable to mild alkaline and acid hydrolysis

The combined solvent layers containing the alkali- and acid-stable phospholipids were taken to near dryness *in vacuo*. This operation was conveniently performed by evaporating successive small portions at 100°, in a drawn-out thick-walled test tube (Fig. 1). The final solvent removal was effected by inserting the end of a thick-walled piece of pressure tubing against the tapering neck of the tube and applying a vacuum (Fig. 1). To the residue of lipids and trichloroacetic acid was added 1·25 ml. of dry methanolic 1·7N-HCl. The tube was sealed off in ice and then left in an oven at 100–102° for at least 4 hr. The hydrolysis tube was shielded as a precaution against a pressure explosion and

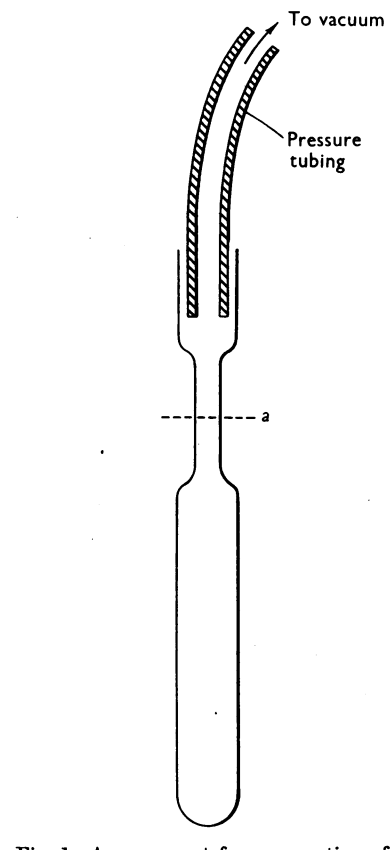

Fig. 1. Arrangement for evaporation of solvent from hydrolysis tube.

cooled well before opening at line '*a*' (Fig. 1). The pressure tubing was now applied over the tapering neck and the methanolic HCl removed *in vacuo*. To the hydrolysed lipids and residual methyl trichloroacetate (0·05 ml.) was added 0·45 ml. of 80% ethanol (C). (Some commercial samples of trichloroacetic acid give residues greater than 0·05 ml. and should be rejected.)

Paper chromatography and ionophoresis

Samples from the three hydrolysates were quantitatively spotted with an Ostwald pipette onto Whatman no. 1 paper which had previously been well washed with 2N-acetic acid and water. Spots were confined to an area of about 1·5–2·0 cm. diameter.

With the hydrolysate of the alkali-labile phospholipids (A) a 0·2 ml. sample was used, and a descending or ascending chromatogram was developed for 14–18 hr. with phenol saturated with water–acetic acid–ethanol (100:10:12, by vol.). For successful chromatography it was found essential to prepare the solvent freshly for each run. If the chromatogram was to be developed in a second dimension it was allowed to dry at room temperature and then washed twice with technical ether to remove phenol. The second solvent used was freshly prepared: methanol–98% formic acid–water (80:13:7, by vol.; ascending chromatography, 8 hr.); alternatively, separation in the second dimension was carried out by ionophoresis. Volatile buffer at pH 3·6 (pyridine–acetic acid–water; 1:10:89, by vol.) was applied to the paper and the phosphate esters were separated by ionophoresis at 2000 v (55 min.) under water-cooled toluene in an apparatus similar to that described by Ryle, Sanger, Smith & Kitai (1955).

The phosphate esters in a 0·3 ml. sample of the hydrolysate of the plasmalogens (B) were separated on a unidimensional paper chromatogram with either the same phenol–acetic acid–ethanol solvent or phenol saturated with water. The hydrolysate of the phospholipids which were stable to mild alkaline and acid hydrolysis (0·2 ml., C) was chromatographed with phenol saturated with water. After unidimensional chromatography the chromatograms were dried in an oven at 60–75°.

Detection of phosphorus-containing spots

A preliminary spray of the dried chromatograms with 0·25% of ninhydrin in acetone followed by heating at 100° for 3 min. assisted in the location of glycerylphosphorylethanolamine and its serine analogue. However, this was not used in the routine analytical procedure. The phosphorus-containing hydrolysis products were located by using the acid molybdate spray of Hanes & Isherwood (1949) followed by irradiation with u.v. light. However, for alkaline hydrolysates containing sodium formate (method I) the spraying reagent was modified and contained 10 ml. of 72% (w/w) perchloric acid, 20 ml. of 5N-HCl, 40 ml. of 5% ammonium molybdate solution and 130 ml. of water. Although this results in slower development of the spots it largely prevents the appearance on the chromatogram of a large greenish blue spot due to the alkali metal. After spraying, the chromatogram was thoroughly dried in a stream of air. The spots were then developed by exposure to a strong source of u.v. (a Hanovia Chromatolite u.v. lamp with the glass filter removed). Very slow development of the spots was found to be due to too much acid remaining in the paper from the acid molybdate spraying.

Estimation of phosphorus in spots

Each spot, plus a generous 0·5 cm. border, was cut out of the chromatogram, weighed on a torsion balance and introduced into a Pyrex boiling tube. Perchloric acid (72%, w/w) was added, the amount depending on the weight of paper spot to be oxidized (1–2 ml.). The tube was heated on an electrical digestion rack suitably protected against possible explosions (many thousands of such oxidations have been performed in this Laboratory with no such occurrence). If 72% perchloric acid was used (but not with 60%) the digestion fluid became colourless within 5–10 min. the oxidation being catalysed by the molybdate in the paper. At this stage the perchloric acid was evaporated by strong heating and reduced in volume as judged by eye to 0·6–0·7 ml. The estimation of phosphorus is independent of perchloric acid volumes between 0·5 and 0·9 ml. When cool the digestion fluid was quantitatively transferred to a tube graduated at 10 ml., with about 8 ml. of water. If a slight precipitate was present, this was removed by heating the tube for 10 min. at 100°. The tube was then cooled to room temperature [the colour development is dependent on temperature (Allen, 1940)]; 0·5 ml. of 5% ammonium molybdate was added and, after mixing, 0·4 ml. of reducing reagent (Fiske & Subbarow, 1925). The solution was made up to the 10 ml. mark, mixed and allowed to stand for 20 min. and the colour read at 660 mμ. Suitable weighed paper blanks were used, and the actual blank for each spot was calculated from its weight compared with the weight and colour reading of the blank: such blanks were usually low (*E* 0·01–0·03).

Calculation of phospholipid concentrations

For the samples and dilutions used in the above procedure the following calculations were applied to determine the percentage of phosphorus from a given phospholipid in the total phospholipid mixture. If P_T is the total phospholipid phosphorus hydrolysed and P_X the phosphorus in the spot being considered, the percentage of an alkali-labile phospholipid

$$=\frac{P_X}{P_T}\times\frac{1}{0{\cdot}2}\times 100.$$

For some phospholipids in this group it was necessary to apply a correction to allow for the further breakdown of the deacylated phospholipid derivatives. Thus certain of the glycerylphosphoryl derivatives can be partially broken down during alkaline hydrolysis to form cyclic glycerophosphoric acid (Maruo & Benson, 1959). Under the conditions of alkaline hydrolysis used, about 6% of the glycerylphosphorylcholine formed from lecithin was converted into the cyclic glycerophosphoric acid (6·1, 5·2, 6·1%), so the phosphorus found in the glycerylphosphorylcholine spot was multiplied by 1·06. Similarly, the phosphorus of the combined glycerylphosphorylinositol and phosphorylinositol spot derived from monophosphoinositide was multiplied by 1·40 to allow for the cyclic glycerophosphoric acid and other phosphorus-containing hydrolysis products also formed (see below). On unidimensional chromatograms the glycerylphosphorylethanolamine spot ran to the same position as the cyclic glycerophosphoric acid spot (Fig. 2). Consequently, a correction was made by subtracting the calculated amount of cyclic glycerophosphoric acid formed by the hydrolysis of lecithin and monophosphoinositide

present, i.e. 6% of the glycerylphosphorylcholine phosphorus and 24% of the combined glycerylphosphorylinositol and phosphorylinositol phosphorus.

Percentage of an alkali-stable acid-labile phospholipid (plasmalogen)

$$= \frac{P_X}{P_T} \times \frac{0{\cdot}8}{0{\cdot}3} \times \frac{2}{1{\cdot}6} \times 100.$$

Percentage of a phospholipid stable to mild acid and alkaline hydrolysis

$$= \frac{P_X}{P_T} \times \frac{0{\cdot}5}{0{\cdot}2} \times \frac{2}{1{\cdot}6} \times 100.$$

Micro-modification of the method

A modification with 100–200 μg. of lipid phosphorus enabled a phospholipid distribution to be obtained on small samples of tissue (50–200 mg. wet wt.). The procedure was the same as described in the macro-method except that the mild alkaline hydrolysis was performed with 0·4 N-NaOH in place of the 1 N-NaOH. For chromatography 0·5 ml. of the mild-alkaline hydrolysate (A) was used and all of the hydrolysate B (plasmalogens) and C (lipids stable to mild acid and alkaline hydrolysis). The volume of these samples was decreased for convenient application to the paper, by evaporating *in vacuo* in small glass flasks (10 ml.) drawn to a point at the bottom. Transference of the hydrolysate from the flask to the paper was facilitated by colouring it with a little bromothymol blue. This ran to the solvent front on subsequent chromatography in the phenol solvent but was readily distinguishable from the glycerylphosphorylcholine spot.

Recoveries of the lipid phosphorus hydrolysed tended to be low (80–90%). Since, however, the losses can largely be attributed to non-quantitative transference of the hydrolysates to the paper, the percentage distribution of phospholipids can be calculated with little error by expressing the recovery from the spots as a percentage of the total phosphorus recovered rather than of that hydrolysed as in the macro-method.

Materials

Ovolecithin and monophosphoinositide were prepared by previously described methods (Dawson, 1958*b*). Phosphatidylethanolamine was prepared by fractionating yeast kephalin on a silicic acid column according to Lea, Rhodes & Stoll (1955). Phosphatidylserine and diphosphoinositide were prepared from ox brain by the methods of Folch (1949*a*, *b*). Cardiolipin was donated by Dr M. G. Macfarlane of the Lister Institute and sphingomyelin by Dr J. Olley of the Torry Research Station.

Sodium-α-glycerophosphate was a preparation supplied by Boots Ltd., sodium inositol monophosphate was given by Dr J. N. Hawthorne, Birmingham, and brucine cyclic glycerol 1:2-phosphate by Dr D. Brown, Cambridge.

RESULTS

Mild alkaline hydrolysis of phospholipids. In initial experiments attempts were made to deacylate the phospholipids with volatile bases, e.g. ammonia or diethylamine. However, only very slow hydrolysis of the fatty-acyl-ester bonds was achieved. When sodium hydroxide was used as the base it was found that, provided that slight excess of alkali was present at the end of the hydrolysis, complete deacylation of the alkali-labile phospholipids as measured by appearance of acid-soluble phosphorus or by maximal loss of acyl-ester bonds occurred within 20 min. at 37°. In the present method the sodium hydroxide concentration was reduced to a point which just ensured that with normal lipid extracts there would be slight excess present at the end of the hydrolysis.

This excess was 'neutralized' with ethyl formate, which automatically protects the system against acidification beyond the neutral point, which would cause hydrolysis of the plasmalogens. The resultant sodium formate in the hydrolysate forms a large alkali spot on the chromatogram, which although not affecting the quantitative recovery of phosphorus from the spots does tend to distort two-dimensional chromatograms somewhat, by obscuring the cyclic glycerophosphate spot. However, by using Amberlite IRC 50 resin to remove the alkali (method II) this difficulty can be avoided, but in this case the non-alkali-labile lipids are partly removed by the resin and cannot be subsequently examined.

Hydrolysis of plasmalogens. It is now generally recognized that after alkaline hydrolysis the aldehyde grouping of the plasmalogens becomes extremely labile to acid hydrolysis (Feulgen & Bersin, 1939; Schmidt, Benotti, Herschman & Thannhauser, 1946; Schmidt, Ottenstein & Bessman, 1953). This observation was applied in the present method, the water-soluble products formed on acid hydrolysis being examined by paper chromatography. Trichloroacetic acid was chosen as the acid for the hydrolysis because it could be subsequently removed from the aqueous hydrolysate by ether extraction.

Two lipid fractions rich in plasmalogen were investigated in preliminary experiments. These were ram-spermatozoa phospholipids rich in choline plasmalogen (Lovern, Olley, Hartree & Mann, 1957; the present experiments indicated that some ethanolamine plasmalogen was also present) and brain phospholipids which contain ethanolamine plasmalogen and to a less extent serine plasmalogen (Klenk & Böhm, 1951). The alkali-stable phospholipids from these sources were dissolved in *iso*butanol–chloroform (1:2, v/v) and shaken vigorously with 10% trichloroacetic acid solution at 37°. Maximum liberation of water-soluble phosphorus occurred within 20 min. Paper chromatography showed that this phosphorus was in the form of the water-soluble glycerylphosphoryl derivatives of each plasmalogen base (i.e. glycerylphosphoryl-choline, -ethanolamine and -serine).

Hydrolysis of phospholipids resistant to mild acid and alkaline hydrolysis. Although the phosphorus

in this fraction is commonly used as a measure of sphingomyelin a number of workers have pointed out that the alkali- and acid-stable lipids of tissues contain other phospholipids (Brante, 1949; Mallov, McKibbin & Robb, 1953). Dawson (1954*b*) found that the alkali- and acid-stable phospholipids of brain possessed choline phosphorus molar ratios which were less than one and suggested that sphingomyelin could best be estimated by measuring the choline in the fraction or the difference between total choline and the choline released on mild alkaline and acid hydrolysis. Rennkamp (1949) showed that on the hydrolysis of sphingomyelin with dry methanolic hydrochloric acid, phosphorylcholine and sphingosylphosphorylcholine were produced. These results were recently confirmed by Dawson (1958*a*), who showed that both compounds moved on a phenol–water paper chromatogram as a single fast-running spot. These observations form the basis of the present method, in which that fraction of the phospholipids stable to mild alkaline and acid hydrolysis was hydrolysed for 4 hr. with dry methanolic hydrochloric acid. The total hydrolysate when chromatographed on paper in phenol–water yielded a main spot at about R_F 0·8 and subsidiary spots at lower R_F values. Pure sphingomyelin subjected to the same hydrolytic procedure (mild aqueous alkali and acid followed by methanolic hydrochloric acid) produced a single spot at R_F 0·8. Recently, Ansell & Spanner (1959) have used a similar method to measure the uptake of ^{32}P into brain sphingomyelin.

Paper chromatography of hydrolysates. Fig. 2 shows diagrammatically the approximate position of the phosphorus-containing spots when the three hydrolysates (A, B and C) were chromatographed unidimensionally in phenol-containing solvents. The identities of the spots were determined either by using the pure substances as markers or by hydrolysing the individual phospholipids and determining the position of their hydrolysis products on equivalent chromatograms. A preliminary ninhydrin spray also helped to locate the position of glycerylphosphoryl-ethanolamine and -serine.

In agreement with previous work (Dawson, 1954*a*) it was shown that when pure phosphoglycerides were subjected to mild alkaline hydrolysis small amounts of products other than the simple deacylated phospholipid were formed. Maruo & Benson (1959) have recently identified one of these products as cyclic glycerophosphoric acid. Under the present experimental conditions of alkaline hydrolysis lecithin forms about 6 % of this cyclic compound and monophosphoinositide 20 %: the other phosphoglycerides form very little. The hydrolysis of monophosphoinositide is more complicated than the other phosphoglycerides: although glycerylphosphorylinositol is the predominant product (57 %), the hydrolysate contains cyclic glycerophosphate (20 %), about 14 % of phosphorylinositol and a little glycerophosphate (3 %) as well as 7 % of an unidentified phosphorus-containing hydrolysis product. Thus caution is needed in interpreting the free glycerophosphate in an alkaline hydrolysate of phospholipids as being solely derived from phosphatidic acid. The breakdown of brain inositide was similarly complicated; a number of products were formed, the structure of which is being investigated at present.

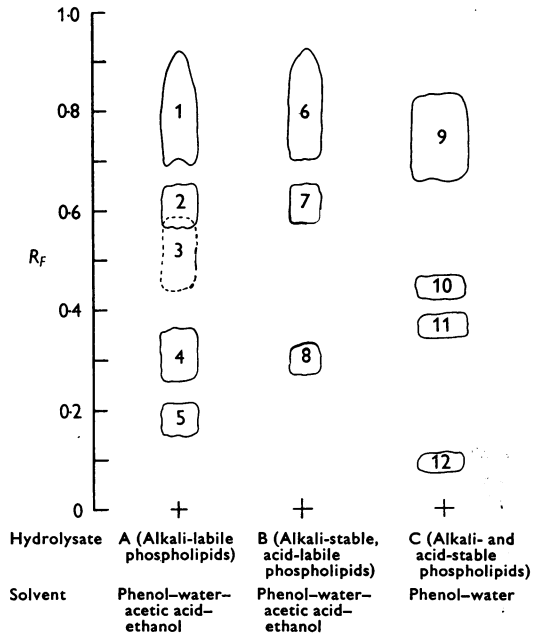

Fig. 2. Unidimensional paper chromatography of phospholipid hydrolysates, showing diagrammatically the approximate form and position of the phosphorus-containing spots. Composition of spots, with parent phospholipid in parenthesis: (1) Glycerylphosphorylcholine (lecithin); (2) glycerylphosphorylethanolamine (phosphatidylethanolamine), cyclic glycerophosphoric acid (lecithin and monophosphoinositide); (3) greenish-blue alkali spot; (4) glycerylphosphorylserine (phosphatidylserine), polyglycerolphosphoric acid (polyglycerolphospholipid), glycerophosphoric acid (phosphatidic acid; monophosphoinositide can give 3 % of glycerophosphoric acid on alkaline hydrolysis); (5) glycerylphosphorylinositol, phosphorylinositol (monophosphoinositide), hydrolysis products of brain inositide; (6) glycerylphosphorylcholine (choline plasmalogen); (7) glycerylphosphorylethanolamine (ethanolamine plasmalogen); (8) glycerylphosphorylserine (serine plasmalogen); (9) phosphorylcholine, sphingosylphosphorylcholine (sphingomyelin); (10), (11), (12) unidentified.

The identities of the minor spots 10, 11 and 12 (Fig. 2) on chromatograms of the hydrolysate of the phospholipids stable to mild alkaline and acid hydrolysis (C) are unknown. The spot 12 with very low R_F (0·09) was usually very faint and occasionally absent from the chromatogram altogether. Dr G. B. Ansell (personal communication) has observed similar spots on the paper chromatography of methanolic hydrochloric acid hydrolysates of the brain phospholipids stable to saponification by the Schmidt *et al.* (1946) procedure. Extracts of sheep-brain and -heart lipids were saponified by the same method (24 hr., 37°, N-sodium hydroxide) and chromatography of the methanolic hydrochloric acid hydrolysate confimed that similar unidentified spots were present. However, although the sphingomyelin phosphorus recovered was very similar to that in the present method of analysis the amount of phosphorus in the unidentified spots was less.

On unidimensional paper chromatography of the hydrolysate of the alkali-labile phospholipids (A), the deacylated products from phosphatidylserine, polyglycerol phospholipid (e.g. cardiolipin) and phosphatidic acid travelled more or less as a single spot (Fig. 2). If separate analysis was required, these compounds were separated by using methanol–formic acid–water as a second solvent or alternatively by using high-voltage ionophoresis at pH 3·6. Ionophoresis, as well as being more rapid, had the additional merit that with brain-lipid extracts it allowed the separation of the deacylation products of mono- and di-phosphoinositide. Fig. 3 shows a tracing of a typical two-dimensional chromatogram obtained with a mild alkaline hydrolysate (A) of sheep-brain phospholipids. Fig. 4 shows a tracing of a single-dimensional chromatogram of the alkaline hydrolysate of sheep-kidney phospholipids which had been subjected to ionophoresis at pH 3·6 in a second dimension. In Table 1 are recorded the R_F values of a number of the hydrolysis products in both the solvents phenol–water–acetic acid–ethanol and methanol–formic acid–water as well as the mobilities on ionophoresis at pH 3·6 in pyridine–acetic acid

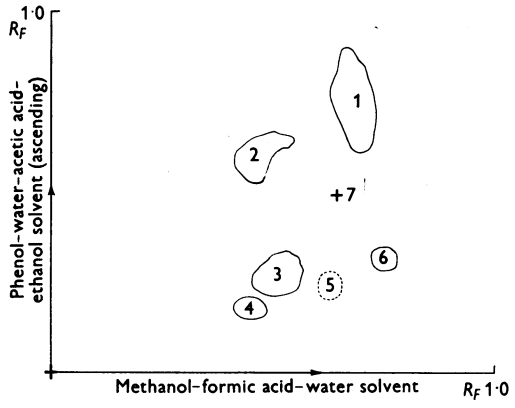

Fig. 3. Tracing of a two-dimensional paper chromatogram of the phosphorus-containing products of mild alkaline hydrolysis of 94 μg. of sheep-brain-phospholipid P. Faint spots are shown in dotted outline. Identity of spots, with parent phospholipid in parenthesis: (1) glycerylphosphorylcholine (lecithin); (2) glycerylphosphorylethanolamine (phosphatidylethanolamine); (3) glycerylphosphorylserine (phosphatidylserine); (4) glycerylphosphorylinositol + phosphorylinositol (monophosphoinositide); (5) polyglycerolphosphate (polyglycerol phospholipid); (6) glycerophosphoric acid (phosphatidic acid?); (7) approximate position of cyclic glycerophosphate (in this chromatogram the alkaline hydrolysate was prepared by method I and the alkali-metal spot obscures the spot).

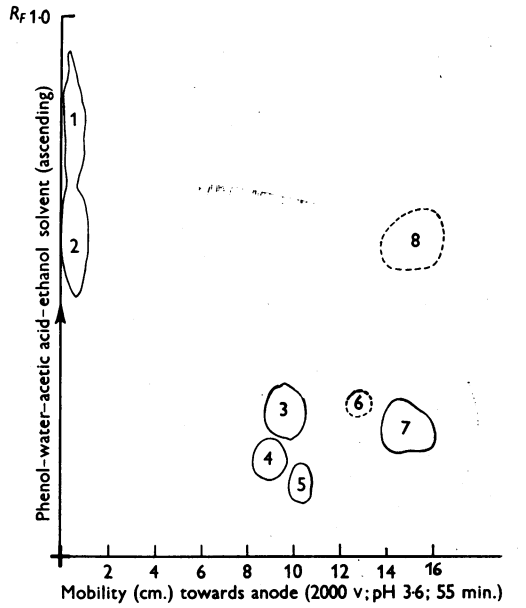

Fig. 4. Tracing of a unidimensional paper chromatogram of the phosphorus-containing products of mild alkaline hydrolysis 108 μg. of sheep-kidney-phospholipid P. The products have been separated in a second dimension with ionophoresis at pH 3·6. Faint spots are shown in dotted outline. Identity of spots, with parent phospholipid in parenthesis: (1) glycerylphosphorylcholine—this spot and spot 2 can be completely separated in descending chromatography (lecithin); (2) glycerylphosphorylethanolamine (phosphatidylethanolamine); (3) glycerylphosphorylserine (phosphatidylserine); (4) glycerylphosphorylinositol (monophosphoinositide); (5) phosphorylinositol (monophosphoinositide); (6) glycerophosphoric acid (phosphatidic acid?); (7) polyglycerolphosphoric acid (polyglycerolphospholipid); (8) cyclic glycerophosphoric acid (lecithin and monophosphoinositide).

Table 1. *Approximate R_F values and ionophoretic mobilities of the phosphorus-containing hydrolysis products produced on the mild alkaline hydrolysis of phospholipids*

Hydrolysis product	Parent phospholipid	R_F: In phenol–water–acetic acid–ethanol	R_F: In methanol–formic acid–water	Mobility at pH 3·6 relative to inorganic P
Glycerylphosphorylcholine	Lecithin	0·77	0·67	0
Glycerylphosphorylethanolamine	Phosphatidylethanolamine	0·63	0·47	0
Glycerylphosphorylserine	Phosphatidylserine	0·30	0·50	0·61
Glycerylphosphorylinositol	Monophosphoinositide	0·20	0·45	0·57
Phosphorylinositol	Monophosphoinositide	0·20	0·45	0·64
Glycerophosphoric acid	Phosphatidic acid	0·33	0·74	0·82
Polyglycerol phosphate	Polyglycerol phospholipid	0·28	0·60	0·96
Hydrolysis products of brain inositide	Brain inositide	0·12	0·66	1·0 and 1·16
Cyclic glycerophosphoric acid	Lecithin / Monophosphoinositide	0·50	0·66	1·0
Inorganic P		0·25	0·85	1·0

Table 2. *Distribution of individual phospholipids in tissues of the sheep*

Tissues were obtained from a ewe (50 kg.) as soon as possible after death (10–40 min.) and frozen in solid CO_2 until used. Results express the phosphorus of the phospholipid as a percentage of the total lipid phosphorus.

	Brain (whole)	Kidney	Liver	Skeletal muscle	Lung	Heart muscle
Total phospholipid (mg. of P/g. of wet tissue)	2·20	1·22	1·94	0·55	1·02	0·91
Phosphatidylcholine (lecithin)	38·6	36·4	55·2	39·9	48·5	27·5
Phosphatidylethanolamine	17·5	20·0	26·5	14·7	14·0	19·1
Phosphatidylserine	16·0	8·2	4·4	3·8	9·9	3·5
Monophosphoinositide	2·2	4·2	6·1	2·6	2·6	3·8
Polyglycerolphospholipid	1·1	6·7	3·2	7·0	0·8	14·5*
'Phosphatidic acid'	1·0	Trace	Trace	—	0·5	—
Choline plasmalogen	—	1·1	—	2·3	0·8	11·4
Ethanolamine plasmalogen	11·0	5·3	0·2	6·6	6·2	6·7
Serine plasmalogen	0·4	Trace	—	—	—	—
Sphingomyelin	9·4	13·2	4·2	6·4	12·4	6·6
Unidentified mild alkali- and acid-stable phospholipid(s)	5·6	3·0	0·5	3·4	1·9	7·9
Recovery (% of total lipid P analysed)	102·8	98·1	100·3	86·7	97·6	101·0

* Cardiolipin.

buffer. It must be emphasized that these R_F values and mobilities can never be regarded as absolute, and can vary slightly from day to day with the length of the chromatographic run, temperature, etc.; nevertheless, the relative positions of the spots remain constant.

Examination of the phospholipids present in sheep tissues. In Table 2 results are presented of the analysis of six tissues of the sheep by the present method. The estimations were performed in duplicate runs showing close agreement. It can be seen that very good recoveries of the lipid phosphorus used for analysis were obtained from the chromatograms, apart from skeletal muscle, where the recovery was 13 % low.

Few previous estimations have been made of the concentrations of individual phospholipids present in sheep tissues. With the alkali-labile phospholipids the distribution of lecithin, phosphatidylethanolamine and phosphatidylserine (Table 2) is closely similar to that found in rat tissues (Dawson, 1957). Although the percentage of phosphorus present in polyglycerol phospholipid was highest in heart (cardiolipin) it is perhaps surprising that there was also an appreciable percentage in both the kidney and muscle lipids and to a lesser extent in liver lipids. McKibbin & Taylor (1952) isolated a polyglycerol phospholipid from horse liver and Dawson (1958*c*) has recently isolated small amounts of a similar compound from rat and guinea-pig liver. A very faint glycerophosphate spot which may correspond to true phosphatidic acid was seen on certain chromatograms. Glycerophosphate is known to be the major product

formed by the mild alkaline hydrolysis of phosphatidic acid (Dawson, 1954*a*; Hübscher & Clark, 1959), but small amounts can also be derived from monophosphoinositide hydrolysis. Hokin & Hokin (1958) recently demonstrated by an isotopic technique the presence of traces of phosphatidic acid in mouse tissues whereas Hübscher & Clark (1959) have isolated about 0·5–1 % of phosphatidic acid from the phospholipids of ox liver.

The analysis of the individual tissue plasmalogens generally agrees with the small amount of evidence at present available on the distribution of tissue plasmalogens obtained by isolation experiments. Thus Feulgen & Bersin (1939) first isolated ethanolamine plasmalogen from muscle and Klenk & Böhm (1951) found that appreciable amounts were also present in brain phospholipids. The present results (Table 2) indicate a similar trend in the tissues of the sheep but also show that ethanolamine plasmalogen is the predominant plasmalogen in the kidneys and lungs. The small amount of plasmalogen in the liver consisted almost entirely of ethanolamine plasmalogen, whereas in heart muscle although choline plasmalogen was predominant, appreciable quantities of ethanolamine plasmalogen were also present. Klenk & Gehrmann (1953) and Rapport & Alonzo (1955) have found that the lecithin fraction of ox heart is rich in choline plasmalogen.

Of the phospholipids stable to mild alkaline and acid hydrolysis, brain, lung and kidney lipids appeared to be the richest sources of sphingomyelin whereas brain and heart-muscle lipids possessed appreciable amounts of phospholipid(s) resistant to alkali and acid hydrolysis, which was not sphingomeylin. In heart muscle this unidentified phospholipid(s) exceeded the amount of sphingomyelin, whereas in liver very little was present.

DISCUSSION

The present method of analysing the phospholipid fraction of a tissue has certain advantages over conventional methods of analysis. The method requires only small amounts of tissue and as only one type of estimation is required (phosphorus) it is rapidly carried through. Further, it enables estimates to be made of phospholipids which have hitherto been detected only by isolation experiments, e.g. cardiolipin, and it resolves the serine, ethanolamine and choline phosphoglycerides into their individual plasmalogen and di-acylated components. No resolution of lecithin and phosphatidylethanolamine from their respective lyso compounds is possible with the present method. In fresh tissues this may not be a serious disadvantage as the amount of lysolecithin appears to be small compared with that of lecithin itself (Marinetti, Witter & Stotz, 1957); in autolysed tissues it may be a more important factor.

The question arises of the identity of the phospholipids which occur in the fraction stable to mild acid and alkaline hydrolysis and which are not sphingomyelin. Carter, Smith & Jones (1958) have recently isolated a new alkali- and acid-stable phospholipid from egg yolk which they identified as the phosphorylethanolamine derivative of batyl alcohol. Evidence was obtained that a similar compound was present in mammalian tissues and it is possible that this would partially account for the present unidentified phospholipid(s).

Comparison of the values in Table 2 shows that the amount of the unidentified phospholipid(s) in a tissue appears to be approximately directly proportional to the total plasmalogen P present in the tissue. This raises the question whether a metabolic relationship exists between the two fractions or if the unidentified lipid represents a type of plasmalogen complex which is not susceptible to mild alkaline and acid hydrolysis.

Note added in proof. Additional evidence has now been obtained that part at least of the unidentified phosphorus in the alkali- and acid-stable fraction is related to the plasmalogens. If the 10 % trichloroacetic acid solution used to hydrolyse the lysoplasmalogens is made 5 mM with respect to mercuric chloride about 20–30 % more water-soluble phosphorus is liberated on shaking at 37° for 30 min. [cf. Schmidt *et al.* (1959) *Amer. J. Dis. Child.* **97**, 691]. This extra phosphorus is derived from the unidentified phosphorus and is released as glycerylphosphoryl-choline or -ethanolamine according to the plasmalogens already present. Work is continuing to determine whether this non-acid-labile plasmalogen represents some different type or combined form of plasmalogen or if it is an artifact of the acid hydrolysis.

SUMMARY

1. A method is presented for the rapid quantitative determination of the individual phospholipids in a small sample of biological material.

2. The products of selective hydrolytic degradation are separated by paper chromatography and their phosphorus contents measured. From these, the nature and amounts of the phospholipids originally present are inferred, appropriate corrections being based upon the hydrolysis of reference compounds.

3. By this method, the distribution of lecithin, phosphatidylethanolamine, phosphatidylserine, monophosphoinositide, polyglycerol phospholipid (cardiolipin), 'phosphatidic acid', choline plasmalogen, ethanolamine plasmalogen, serine plasmalogen and sphingomyelin has been measured in six tissues of the sheep.

I wish to acknowledge the expert technical assistance of Miss N. Hemington.

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Studies in Flavinogenesis

6. THE ROLE OF THREONINE IN RIBOFLAVIN BIOSYNTHESIS IN *EREMOTHECIUM ASHBYII**

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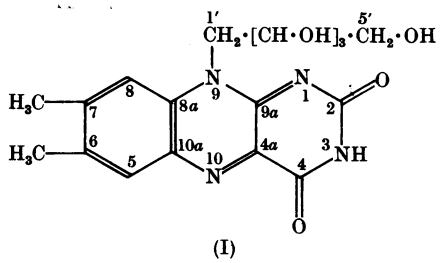
(*Received* 21 *August* 1959)

Goodwin & Pendlington (1954) showed that only two amino acids, L-serine and L-threonine, specifically stimulated the production of riboflavin in the mould *Eremothecium ashbyii*. The mechanism of stimulation by L-serine was elucidated by Goodwin & Jones (1956), using [^{14}C]serine. It had already been shown by Plaut (1954) that C-4*a* and C-9*a* of riboflavin (I) in *Ashbya gossypii* arise from glycine and that C-2 is derived from formate, and Goodwin & Jones demonstrated that C-1 and C-2 of serine were found in C-4*a* and 9*a* of riboflavin and C-3 of serine in C-2 of riboflavin. Thus serine was incorporated into the riboflavin molecule after being split into a C_1 unit (formate) and glycine.



(I)

The mechanism of threonine incorporation has now been studied with L-[^{14}C]threonine. Part of this work has already been briefly reported (Goodwin & Horton, 1959).

EXPERIMENTAL

Organism. Our normal strain of *Eremothecium ashbyii* (Brown, Goodwin & Jones, 1958) was used throughout the investigation.

Medium. The medium used was the basal medium described by Goodwin & Pendlington (1954) with bacteriological peptone added to a concentration of 0·04%. After adjusting to pH 5·8, the medium was autoclaved at 15 lb./in.^{2} for 15 min.

* Part 5. Goodwin & McEvoy (1959).

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