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Improvements in the Method of Determining Individual Phospholipids in a Complex Mixture by Successive Chemical Hydrolyses

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A method whereby the complete phospholipid distribution in a lipid sample could be determined by successive chemical hydrolyses of the lipid, followed by an examination of the water-soluble fragments removed, was described by Dawson (1960). Thus the diacylated phosphoglycerides are hydrolysed with alkali, the plasmalogens with acid and the remaining phospholipids with methanolic hydrochloric acid. During regular use in this Laboratory the method has undergone a number of improvements. In the main these fall into two groups: (1) a more quantitative recovery of the plasmalogens by preventing the reaction by which cyclic acetals are formed on acid hydrolysis (Davenport & Dawson, 1962); (2) coverage of the more-recently-discovered minor phospholipid components, which include phosphatidylglycerol (Benson & Maruo, 1958; Macfarlane, 1961), phosphatidylinositomannosides (e.g. Vilkas & Lederer, 1960), triphosphoinositide (Dittmer & Dawson, 1961; Grado & Ballou, 1961), diacylglycerylphosphorylinositol monophosphate (diphosphoinositide) (Dawson & Dittmer, 1961; Brockerhoff & Ballou, 1961) and alkyl ether ethanolamine phospholipid (Carter, Smith & Jones, 1958; Svennerholm & Thorin, 1960). These improvements, and other minor refinements, are briefly reported in the present paper.

METHODS

Preparation of lipid samples. The method was as described by Dawson (1960). Any proteolipid material in the original extract must be removed by evaporating to dryness *in vacuo* in the presence of water and redissolving the lipid in chloroform or light petroleum. Failure to do this can lead to troublesome emulsification and excessive interfaces during the partition of the hydrolysates between solvent and water. This can result in a low recovery of the individual phospholipids.

Alkaline hydrolysis of acyl ester bonds and separation of the water-soluble phosphate ester. 'Method I' of Dawson (1960) was used. On hard centrifuging of the hydrolysate distributed between chloroform–isobutanol (2:1, v/v) and water the upper and lower phases were usually almost clear and the amount of interfacial material small. When difficulty was experienced in obtaining a clear-cut separation a big improvement was obtained by freezing the emulsion in solid carbon dioxide and thawing before centrifuging or, alternatively, by adding cetyltrimethylammonium bromide (final concn. 0·25%) to the water.

The water-soluble phosphate esters produced were separated two-dimensionally on paper by: (1) descending chromatography in phenol saturated with water–acetic acid–ethanol (50:5:6, by vol.) and (2) ionophoresis at pH 3·6 with volatile buffer [pyridine–acetic acid–water (1:10:89, by vol.)]. Ionophoresis was much preferable to chromatography in the second direction as besides being quicker it gave increased resolution and removed Na^+ ions which interfere with the spray for the detection of phosphate esters. As sodium formate was present in the hydrolysate (produced by neutralizing excess of alkali with ethyl formate) it was essential for good resolution to chromatograph the hydrolysate initially in the freshly prepared phenol solvent before carrying out the ionophoresis. After chromatography in the phenol solvent the dried paper supported on glass rods was wetted with pyridine–acetic acid buffer from a hand-operated bulb pipette. The buffer was finally allowed to flow by capillary action into the theoretical line of phosphate esters which had been separated by the phenol solvent so that this was wetted from both sides. Ionophoresis was carried out under toluene for 1 hr. at 40 v/cm. in an apparatus similar to that described by Ryle, Sanger, Smith & Kitai (1955).

Amino compounds were located by spraying with 0·25% (w/v) ninhydrin in acetone followed by heating the paper at 80° for 5 min. The phosphate esters were then located with the acid molybdate spray of Hanes & Isherwood (1949) followed by irradiation with u.v. light.

Hydrolysis of plasmalogens and separation of the water-soluble phosphate esters. A 1·6 ml. sample of the almost clear lower solvent phase from the above alkaline hydrolysis was transferred to a 10 ml. stoppered centrifuge tube. It was then vigorously shaken at 37° for 30 min. with 0·8 ml. of 1% (w/v) trichloroacetic acid containing $HgCl_2$ (5 mM), and then cooled. Water-saturated ether (2 ml.) was added, and after the mixture had been shaken and centrifuged the upper solvent layer was removed with a Pasteur pipette. The lower aqueous layer was then extracted twice with 2 ml. of water-saturated chloroform–ether–isobutanol (1:1:2, by vol.) and finally with 2 ml. of water-saturated ether. It was then neutralized or made slightly alkaline by holding a wick soaked in dilute ammonia in the air of the tube which was then stoppered and shaken. A sample (0·3 ml.) was spotted on paper and subjected to descending chromatography with phenol–water–acetic acid–ethanol solvent (see above). After the papers had been dried at 60–75° they were sprayed with ninhydrin to locate the glycerylphosphorylethanolamine and glycerylphosphorylserine, and the phosphate esters were detected as above.

Treatment with methanolic hydrochloric acid and separation of products. The combined solvent layers from the hydrolysis of the plasmalogens were evaporated to dryness (Dawson, 1960) and heated in a sealed tube for 4 hr. with 1·25 ml. of water-free methanolic 2N-HCl at 100°. The mixture was evaporated to dryness *in vacuo* and 0·5 ml. of water and 2 ml. of water-saturated ether were added. The mixture was shaken and centrifuged, the upper ether layer was withdrawn and the aqueous layer was re-extracted with 1 ml. of water-saturated ether. A sample (0·2 ml.) of the aqueous layer was subjected to descending chromatography on paper in the same phenol–water–acetic acid–ethanol solvent. The combined ether extracts were evaporated to dryness, and oxidized with perchloric acid to estimate the alkyl ether phospholipid phosphorus as described below.

Determination of phosphorus. The phosphorus in the chromatographic spots was determined as described by Dawson (1960). The digestion of the paper spots with 72% (w/w) perchloric acid was always carried out on an electrical heating rack suitably shielded against possible explosions, although none have occurred during 5 years of operation. In our opinion, to avoid such mishaps, it is necessary to do the digestion in a large enough vessel (boiling tube) and always to add sufficient perchloric acid (1 ml./100 mg. of paper) so that the mix becomes liquid and local overheating cannot occur.

The digestion of the alkyl ether phospholipids (carried out after thorough removal of the ether) tended to be somewhat more troublesome because of the presence of free fatty acid and aldehydes carried through from the procedure. These charred on adding the perchloric acid and were only slowly oxidized with a tendency to spit. The oxidation was appreciably improved by adding 0·05 ml. of 1% (w/v) ammonium molybdate to the 1 ml. of perchloric acid used for the oxidation.

The estimation of the alkyl ether phospholipid P was complicated by the presence of mercury that was carried through from the plasmalogen hydrolysis. This formed a faint cloudiness on adding the Fiske & Subbarow (1925) reagents although it did not do so when P was determined by the method of Allen (1940). The mercury in the digest was simply removed by the following procedure. The perchloric acid digest (0·6–0·7 ml.) was diluted to 6 ml. in a stoppered graduated tube and 0·1 ml. of N-HCl added. The digest was then extracted twice with equal volumes of ether and finally with an equal volume of light petroleum (b.p. 40–60°). The mercury was removed in the solvent layers, presumably as mercuric chloride since failure to add the hydrochloric acid resulted in inefficient extraction. The lower aqueous layer was treated with the Fiske & Subbarow (1925) reagents, diluted to 10 ml. and the colour read at 660 mμ after the solution had stood for 20 min.

Calculation of phospholipid concentrations. The calculations used were as described by Dawson (1960). With a few of the phospholipids it was necessary to apply a small correction to allow for the further hydrolysis of the deacylated derivatives by the alkali. This extra decomposition was constant if the conditions of hydrolysis remained the same. Thus the glycerylphosphorylcholine P was increased by 6% to allow for the formation of cyclic glycerophosphate during the hydrolysis of lecithin. The sum of glycerylphosphorylinositol P and phosphorylinositol P was multiplied by 1·4 to give the phosphatidylinositol P, and the glycerylphosphorylinositol diphosphate P plus the inositol triphosphate P by 1·2 to give the triphosphoinositide P.

With the chromatograms of the solution treated with methanolic HCl (C) the phosphorus in the minor spot at R_F 0·47 (see Fig. 1C, spot 22) was added to that of the plasmalogens and the trace of inorganic phosphate P (spot 21) was added to the joint phosphorylcholine P and sphingosylphosphorylcholine P formed from sphingomyelin.

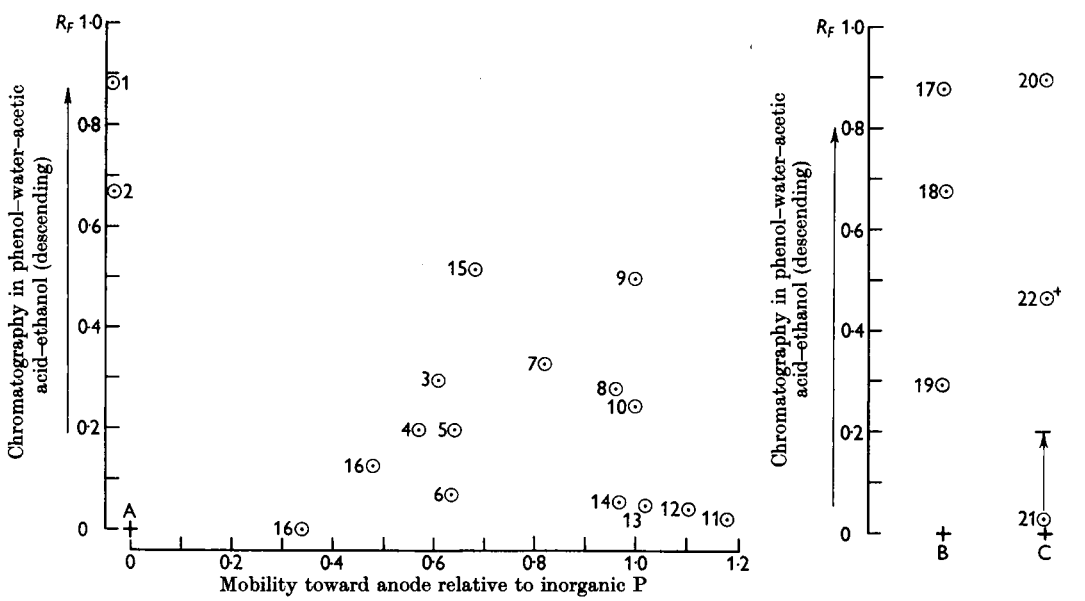

Fig. 1. For legend see facing page.

Fig. 1. Maps of water-soluble phosphate esters in: A, alkaline hydrolysates of phospholipids separated by chromatography and ionophoresis; B, acid–mercuric chloride hydrolysates of the phospholipids stable to mild alkaline hydrolysis separated by chromatography; C, methanolic HCl-treated solutions of the phospholipids stable to mild alkali and acid hydrolysis separated by chromatography. Experimental details are given in the text.

Conditions of successive chemical hydrolyses	No. on map	Compound	Parent phospholipid	Distribution of phospholipid in biological samples
A, 0·03 N-NaOH in ethanol–water (4:1, v/v) at 37° for 20 min.	1	Glycerylphosphorylcholine	Lecithin (lysolecithin)	Ubiquitous, apart from bacteria; lysolecithin reported in blood plasma and traces in other tissues
	2	Glycerylphosphorylethanolamine	Phosphatidylethanolamine	Widely distributed but very low concn. in brain white matter
	3	Glycerylphosphorylserine	Phosphatidylserine	All animal tissues; high concn. in brain and erythrocytes
	4	Glycerylphosphorylinositol	Phosphatidylinositol (monophosphoinositide)	All animal tissues; yeasts; higher plants
	5	Phosphorylinositol		
	6	Unknown (cyclic derivative?)		
	7	Glycerophosphoric acid	Phosphatidic acid (traces can be formed from phosphoinositides)	Traces in animal tissues; presence in higher plants probably due to phospholipase D action
	8	Bis(glycerylphosphoryl)glycerol	Bis(phosphatidyl)glycerol (cardiolipin)	Animal tissues and photosynthetic plants; highest concn. in heart; also concentrated in mitochondria
	9	Cyclic glycerophosphoric acid	Lecithin, phosphatidylinositol	—
	10	Inorganic phosphate	Traces often tenaciously accompany higher phosphoinositides of brain	—
	11	Inositol triphosphate	Triphosphoinositide	Nervous tissue; electric organ; traces in other animal tissues
	12	Glycerylphosphorylinositol diphosphate		
	13	Inositol diphosphate	Diphosphoinositide	Nervous tissue
	14	Glycerylphosphorylinositol monophosphate		
	15	Bis(glyceryl) phosphate	Phosphatidylglycerol	Photosynthetic plants; *M. lysodeikticus*; traces in animal tissues
	16	Deacylation products of phosphatidyl inositotrimannoside (the slowest running is predominant)	Phosphatidylinositotrimannoside*	Mycobacteria
B, 10% (w/v) Trichloroacetic acid containing $HgCl_2$ (5 mM) at 37° for 30 min.	17	Glycerylphosphorylcholine	Choline plasmalogen	Many animal tissues, mammalian spermatozoa; highest concn. in heart; very low in brain and liver
	18	Glycerylphosphorylethanolamine	Ethanolamine plasmalogen	Many animal tissues, marine invertebrates; highest concn. in brain, low in liver
	19	Glycerylphosphorylserine	Serine plasmalogen	Low concn. in brain and blood
C, Methanolic 2 N-HCl at 105° for 4 hr.	20	Phosphorylcholine + sphingosylphosphorylcholine	Sphingomyelin	Widely distributed, but not in bacteria
	21	Inorganic phosphate		
	22†	Glycerophosphate derivative (?)	Cyclic acetal plasmalogens mostly formed from natural plasmalogens by acid–$HgCl_2$ hydrolysis	Some may be present in marine invertebrates

* It is likely that the deacylation products of other phosphatidyl inositomannosides would occupy a similar position.
† Not the cyclic 1,2-phosphate nor the methyl esters; sometimes free glycerophosphate (R_F 0·33) is formed as well.

Table 1. *Distribution of individual phospholipids in various tissues*

Tissues were obtained from freshly slaughtered animals and carried to the laboratory on ice. Erythrocyte stroma were prepared from 5-day-old human blood containing citrate by Dr I. M. Glynn of the Physiology Department, University of Cambridge (Dunham & Glynn, 1961). Results express the P of the phospholipid as a percentage of the total lipid P. Experimental details are given in the text.

	Human erythrocyte stroma	Ox heart	Ox liver	Ox brain
Phosphatidylcholine	33·5	24·2	54·2	29·2
Phosphatidylethanolamine	17·6	16·5	9·4	12·1
Phosphatidylserine	14·3	2·4	4·2	16·6
Phosphatidylinositol	0	4·1	7·9	3·2
Phosphatidic acid	2·2	0	2·2	0·5
Cardiolipin	0	8·9	4·1	0·7
Choline plasmalogen	1·2	17·5	1·5	0
Ethanolamine plasmalogen	10·4	11·0	3·6	21·1
Serine plasmalogen	0	0	0	Trace
Sphingomyelin	20·1	11·5	5·8	12·5
Alkyl ether phospholipid	1·5		0·5	2·1
Recovery (% of total lipid P analysed)	100·8	96·1	93·4	98·0

MATERIALS

Triphosphoinositide was prepared by the method of Dittmer & Dawson (1961). We wish to acknowledge gifts of phosphatidyl glycerol from Dr M. G. Macfarlane, calf-brain alkyl ether phospholipid from Dr L. Svennerholm and phosphatidyl inositotrimannoside from Dr E. Lederer.

RESULTS

Fig. 1A shows a map of the water-soluble phosphate esters which have been detected in mild alkaline hydrolysates of phospholipids and opposite this is a table giving the parent phospholipids and their known distribution in biological samples. The glycerylphosphorylserine and glycerylphosphorylethanolamine can be located by a preliminary spray with ninhydrin before the phosphorus-detecting spray and serve as suitable points of reference. Likewise inorganic phosphate, when present or if added as a marker, gives an initial yellow colour with the phosphorus-detecting spray.

Fig. 1B shows the position on phenol chromatograms of the water-soluble phosphate esters produced by the acid–mercuric chloride hydrolysis of the lysoplasmalogens. It was found desirable to confirm the presence of glycerylphosphorylserine and glycerylphosphorylethanolamine in the acid–mercuric chloride hydrolysate (B) by spraying the chromatogram with ninhydrin before the phosphate esters were located.

Fig. 1C shows the position on a unidimensional phenol chromatogram of the water-soluble phosphate esters formed by the treatment with methanolic hydrochloric acid of the phospholipids stable to mild alkaline and acid hydrolysis. The minor spot at position 21 was identified as inorganic phosphate formed by the further hydrolysis of the phosphorylcholine liberated from sphingomyelin. The other minor spot with R_F 0·47 (22) is a glycerophosphate derivative arising from breakdown of traces of the cyclic acetal phospholipid still formed from the lysoplasmalogens during the acid–mercuric chloride hydrolysis. Thus when the fraction stable to mild alkaline and acid–mercuric chloride hydrolysis was hydrolysed in 1% (w/v) mercuric chloride at 100° for 1 hr. (Thannhauser, Boncoddo & Schmidt, 1951) glycerylphosphorylcholine or glycerylphosphorylethanolamine was liberated in proportion to the plasmalogens present in the original lipid sample. The hydrolysis also liberated much phosphorylcholine from the sphingomyelin present in the fraction.

The alkyl ether phospholipid P remained soluble in lipid solvents throughout the entire procedure owing to the long-chain alkyl ether grouping which is very stable to hydrolysis. It accumulated in the final ether solution and was estimated after removal of the Hg^{2+} ions which interfered with the determination of phosphorus (see the Methods section).

Table 1 presents the complete phospholipid distribution in the phospholipids extracted from human erythrocyte stroma and the liver, heart and brain of the ox. Recovery of the total lipid and P from the individual hydrolysis products ranged from 93·4 to 100·8% of the phospholipid P taken, and this result is typical. The brain phospholipid had been extracted with neutral chloroform–methanol and therefore it did not contain higher phosphoinositides such as triphosphoinositide (Dittmer & Dawson, 1961).

SUMMARY

1. Modifications are described of the method (Dawson, 1960) of determining individual phos-

pholipids in a complex mixture by successive chemical hydrolyses.

2. These extend its coverage to include phosphatidylglycerol, phosphatidylinositomannosides, the higher phosphoinositides and the alkyl ether phospholipids.

3. The recovery of individual plasmalogens is greatly improved by hydrolysing them to glyceryl-phosphoryl-base derivatives with a trichloroacetic acid–mercuric chloride reagent, which reduces the formation of cyclic acetals to a minimum.

4. Complete analyses are presented for the phospholipid distribution in human erythrocyte stroma and ox heart, liver and brain.

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The Accumulation of Salicylic Acid by Mycobacteria During Growth on an Iron-Deficient Medium

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Cultures of *Mycobacterium smegmatis* contain a number of fluorescent substances, most of which have not been identified. When growth of the organism takes place under conditions of iron deficiency the formation of a compound with a purple–blue fluorescence is particularly noticeable.

This paper describes the identification of this compound as salicylic acid, and gives the concentrations found in cultures of some species of *Mycobacterium* grown under various conditions.

Salicylic acid has been found previously in small amounts in the acetone-soluble lipid fraction of *M. tuberculosis*, where it is accompanied by phenylacetic acid (Stendal, 1934). Anisic acid, phthalic acid and phenethyl alcohol also have been isolated from lipid extracted from the same organism (Edens, Creighton & Anderson, 1944; Aebi, Asselineau & Lederer, 1953; Goris & Sebetay, 1946). There is no evidence about the origin or function of these simple aromatic substances in *M. tuberculosis*.

EXPERIMENTAL

Organisms. The strain of *Mycobacterium smegmatis* is of unknown origin (Winder & O'Hara, 1962). *Mycobacterium phlei* (N.C.T.C. 525) was obtained from the National Collection of Type Cultures.

Mycobacterium tuberculosis BCG was obtained from Dr O. Sievers, BCG Laboratory, Sahlgrens Hospital, Gothenburg S.V., Sweden.

Medium. The Proskauer and Beck medium used throughout contained: asparagine, 5·0 g.; magnesium citrate, 1·5 g.; KH_2PO_4, 5·0 g.; K_2SO_4, 0·5 g.; glycerol, 20·0 ml.; de-ionized water, 1 l. The pH was adjusted to 7·0 with 10N-NaOH for the growth of *M. smegmatis* and *M. phlei*, and to 6·8 for the growth of *M. tuberculosis*. The medium was depleted of trace metals by autoclaving with alumina, as described by Winder & O'Hara (1962). Metals were added to the depleted medium to give the following concentrations (per ml.): in normal medium, 2·3 μg. of Fe^{3+} and 0·46 μg. of Zn^{2+} ions; in iron-deficient medium, 0·43 μg. of Fe^{3+} and 0·46 μg. of Zn^{2+} ions; in zinc-deficient medium, 2·3 μg. of Fe^{3+} and 0·06 μg. of Zn^{2+} ions.