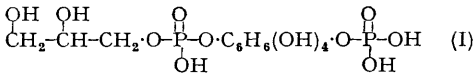
698. *The Structure of the Glycerinositophosphatide of Ground-nut.*

By T. Malkin and A. G. Poole.

By means of paper-chromatographic checks during fractionation, the inositol-containing phosphatide of ground-nuts, which comprises some 38% of the phosphatides present, has been isolated in a high degree of purity. It differs from other known phosphatides in having a nitrogen–phosphorus ratio of 1 : 2, and its degradation products are not inconsistent with structure (II), namely, the *N*-glycosyl derivative of the ethanolamine ester of phosphatidylinositol phosphate. Each molecule of phosphatide contains two molecules of L-arabinose and one of D-galactose. Mild hydrolysis gives a disaccharide of arabinose and galactose, but the detailed arrangement of the three sugar molecules is not yet known.

The presence of inositol in phospholipids was first noted by Anderson (*J. Amer. Chem. Soc.*, 1930, **52**, 1607) in tubercle lipids (cf. Anderson and Roberts, *ibid.*, p. 5023; Cason and Anderson, *J. Biol. Chem.*, 1938, **126**, 527; de Sütö-Nagy, and Anderson, *ibid.*, 1947, **171**, 761). It has also been found in soya-bean lecithin by Klenk and Sakai (*Z. physiol. Chem.*, 1939, **258**, 33) and by Woolley (*J. Biol. Chem.*, 1943, **147**, 581), in ox-brain kephalin by Folch and Woolley (*ibid.*, 1942, **142**, 963; cf. Folch, *ibid.*, 1949, **177**, 505), and in ground-nut kephalin by Hutt, Malkin, Poole, and Watt (*Nature*, 1950, **165**, 314).

Little is known concerning the structure of inositol phospholipids, and the evidence is too slender even to decide whether the various workers were dealing with the same compounds; but there are common features in the papers mentioned which suggest the existence of a phosphatide, or a closely related group of phosphatides, based on the glycerol ester (I) of inositol diphosphate.



(I)

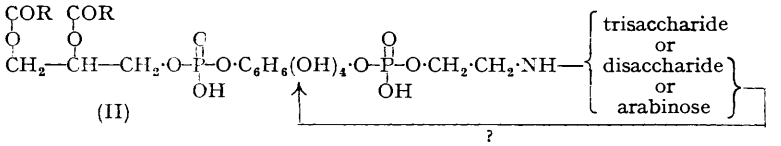
Thus, on hydrolysis of the various phosphatides, Klenk and Sakai found inositol and glycerol monophosphates; de Sütö-Nagy, and Anderson found an acid $C_9H_{20}O_{14}P_2$, which on further hydrolysis gave equimolecular amounts of the same two monophosphates; Folch found equimolecular amounts of inositol "*meta*"-diphosphate and glycerol, and Woolley found inositol phosphate.

Continuing our earlier work (Hutt *et al.*, *loc. cit.*) we have now obtained a more homogeneous fraction by carrying out chromatographic checks during the fractional precipitations of the mixed phosphatides, in order to ensure that only one nitrogenous base was present. In this way, we have obtained a product, some 38% of the ground-nut phosphatide, which contains ethanolamine as the only base. It is a colourless powder, which darkens slightly on storage and responds to the Scherer test for inositol, the acraldehyde test for glycerol, and the Molisch test for carbohydrates; but it does not reduce Fehling's solution until after hydrolysis with dilute mineral acid, *i.e.*, the sugars are combined through glycosidic groups. The nitrogen–phosphorus ratio is 1 : 2, in contrast to that of other known phosphatides—lecithin and kephalin 1 : 1, sphingomyelin 2 : 1.

Analysis of the acid hydrolysis products of this glycerinositophosphatide by paper partition chromatography showed the presence of α- and β-glycerophosphoric acid, inositophosphoric acid, ethanolamine, ethanolamine phosphate, arabinose, and a disaccharide which could be further hydrolysed to arabinose and galactose. The glycerol and inositol phosphates were isolated and found to be in the molecular ratio 1 : 1, and the parent inositol was found to be the inactive form, m. p. 222°. Estimated by Hirst and Jones's method (*J.*, 1948, 1679; 1949, 1659), the molecular ratio of arabinose to galactose was 2 : 1. The two sugars were identified as L-arabinose and D-galactose.

Alkaline hydrolysis of the phospholipid yielded some 40% (by weight) of fatty acids. These were not examined exhaustively, but in agreement with Hilditch and Zaky's report (*Biochem. J.*, 1942, **36**, 815) they were found to be mainly saturated and unsaturated C_{16} and C_{18} acids.

These results would agree with a structure (II) for the glycerinositophosphatide, but we found nitrogen and phosphorus values too high, and the acetyl value too low, indicating a lower sugar content. It is possible that the sugar-free phosphatide occurs naturally, as such, or that the sugar may have been lost by enzyme action in the ground-nut or during extraction of the phosphatide.



(II)

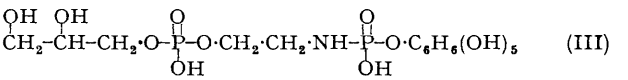
R·CO = mainly palmitoyl, stearoyl, oleoyl, linoleoyl.

In order to remove the analytical uncertainty due to the presence of an indefinite mixture of fatty acids, we removed the latter by alkaline alcoholysis, and after a purification by means of neutral and basic lead acetates we obtained a homogeneous product, which on chromatographic analysis in three solvent mixtures gave a single distinct spot when tested either for sugars or phosphates.

This product is a white crystalline solid, which is very hygroscopic and extremely insoluble in alcohol. It responds to the Molisch, Scherer, and acraldehyde tests, but does not give Fehling's or the ninhydrin test until after hydrolysis with dilute mineral acid.

Its analyses are correct for (II), with the fatty acid radicals removed, and its equivalent weight is 444 (alkali titration; theor. mol. wt., 883). Titration to light-screened methyl red showed that the phosphate groups are in the same state of acidity. Examination of the acid hydrolysis products showed that glycerol and inositol monophosphates were present in the ratio 1 : 1, and arabinose and galactose in the ratio 2 : 1.

As one of us (A. G. P.) has had to discontinue this work, it is reported with a few points still undecided. Thus, although Folch isolated inositol "*meta*"-diphosphate, the positions of the phosphate groups in the original phosphatide are uncertain, owing to the possibility of migration of the phosphate groups during hydrolysis. Again, the negative ninhydrin test before hydrolysis suggests presence of a *N*-glycosyl derivative; but it has still to be determined whether the sugars are present as a trisaccharide, or as a mono- and di-saccharide with one of the sugars combined with inositol. Other possible explanations



(III)

of the negative ninhydrin test are not excluded, *e.g.*, structure (III) with the sugars attached to inositol, although this could not give rise to inositol diphosphate. The above points are under investigation.

Experimental

Our raw material was commercial ground-nut "lecithin," kindly provided by Messrs. J. Bibby & Sons, Ltd. It is a dark viscous oil, which for technical purposes contains a quantity of refined vegetable oil. This was removed as follows: A solution of crude "lecithin" (100 g.) in ether (200 c.c.) was poured, with stirring, into ten times its volume of acetone. The precipitate was allowed to settle overnight, and the mother-liquor was decanted. The somewhat sticky residue was stirred with acetone (400 c.c.) until it became more granular, and was filtered off. Two repetitions of this process yielded 62 g. of a dark brown, friable but slightly sticky, solid.

Isolation of the "Kephalin" Fraction.—The above phospholipid mixture (100 g.), dissolved in ether (200 c.c.), was poured slowly into ethanol (1 l.) with vigorous stirring. After 12 hr., the precipitate was filtered off and washed with 5 : 1 ethanol–ether, followed by ethanol. The solvent was removed *in vacuo*, and the whole process was repeated twice. The product was a light brown friable powder (63·2 g.).

Determination of the Bases.—After first hydrolysing the phospholipid fraction as described below, we used the chromatographic method of Chargaff, Levene, and Green (*J. Biol. Chem.*, 1948, **175**, 67). 50 mg. of material were emulsified with 5 c.c. of 6N-hydrochloric acid, and refluxed for 12 hr. After cooling overnight in the refrigerator, the solid cake of fatty acids which separated was filtered off, and the filtrate was evaporated to dryness *in vacuo* over potassium hydroxide. The residue was dissolved in a few drops of water, and the resulting syrup was used for the chromatographic analysis.

The hydrolysate of the above "kephalin" fraction contained ethanolamine phosphate, choline chloride, ethanolamine hydrochloride, and serine (R_F 0·02, 0·11, 0·32, and 0·55 respectively). This so-called "kephalin" is therefore a complex mixture.

Isolation of Glycerinositophosphatide (X).—We adopted the method used by Folch (*J. Biol. Chem.*, 1942, **146**, 35) who has shown that fractional precipitation from chloroform solution by ethanol is an effective method for separating phospholipid mixtures.

To the above "kephalin" (20 g.) in chloroform (160 c.c.), ethanol (180 c.c.) was added with vigorous stirring. The initial turbidity cleared gradually, leaving a viscous lower layer, from which the clear upper layer was decanted. The viscous layer was then stirred with ethanol (50 c.c.), and the solid which separated was filtered off, washed with ethanol, and dried *in vacuo* (P_2O_5), giving fraction I (14·7 g.).

Ethanol (55 c.c.) was added with stirring to the supernatant liquid from fraction I. A viscous layer separated overnight, and was treated as above, giving fraction II (1·8 g.).

The mother-liquor from fraction II was poured, with stirring, into ethanol (500 c.c.) and the solid which separated overnight was filtered off, washed with ethanol, and dried *in vacuo*, giving fraction III (1 g.).

On concentration of the mother-liquor from fraction III to half its volume and cooling in the refrigerator overnight, a small amount of solid separated. After filtration, washing with ethanol, and drying, fraction IV remained as a slightly sticky powder (0·23 g.).

The filtrate from fraction IV was concentrated to 20 c.c. and poured into acetone (100 c.c.). A yellowish pasty material, which could not be filtered, was precipitated. After decantation of the mother-liquor, trituration with one or two changes of acetone, and removal of solvent there remained fraction V (0·15 g.).

The various fractions were submitted to Molisch, Scherer, and chromatographic tests with the results recorded in the Table. These show that fraction I contains the bulk of the glycerinositophosphatide together with a little phosphatidylserine. The latter is concentrated in fraction III, whilst fractions IV and V contain mainly phosphatidyl-choline and -ethanolamine (the classical lecithin and kephalin).

Fraction	Molisch	Scherer	Bases present	Concn.
I	Strong	Strong	Serine	Weak
			Ethanolamine HCl	Strong
			,, phosphate	,,
II	,,	,,	Serine	,,
			Ethanolamine HCl	Weak
			,, phosphate	,,
III	Weak	Weak	Serine	Strong
			Ethanolamine HCl	Weak
			,, phosphate	V. weak
IV	Negative	Negative	Serine	Weak
			Choline chloride	Strong
			Ethanolamine HCl	,,
V	,,	,,	Choline chloride	,,
			Ethanolamine HCl	,,

Further purification of fraction I. Fraction I (20 g.), dissolved in chloroform (200 c.c.), was poured with vigorous stirring into ethanol (250 c.c.). The viscous layer which gradually separated was triturated with ethanol, to bring about solidification, and filtered off. This process was carried out five times and the solid residue was then dried *in vacuo* (yield, 17·3 g.). In order to remove possible inorganic impurities, this product was submitted to dialysis as follows. The phospholipid (10 g.) was emulsified with water (100 c.c.) and placed in a Cellophane container surrounded by distilled water. The latter was changed every 12 hr. for 2 days. The emulsion was then concentrated to 10 c.c. under reduced pressure at 40°; frothing prevented further concentration. Final drying was carried out at −50°/0·1 mm., to yield a fine white powder which, after trituration with acetone (20 c.c.), was filtered off at the pump and dried *in vacuo*, giving 9·3 g., m. p. 209—210° (decomp.). The only nitrogenous bases present in the hydrolysate of this material were ethanolamine and ethanolamine phosphate. The final yield of glycerinositophosphatide (X) is *ca.* 60% of the crude kephalin (38% of the total ground-nut phosphatide).

General Properties of the Above Glycerinositophosphatide (X).—The material (X) is a pale brown, friable solid, soluble in chloroform, benzene, and to some extent in ether (not if sodium-dried). The solubility in all these solvents is increased by the addition of a few drops of water. It is insoluble in acetone, ethanol, and cold glacial acetic acid, but dissolves in hot acetic acid. It gives the Molisch, Scherer, and acraldehyde test, but does not reduce Fehling's solution until after hydrolysis with dilute mineral acid. When ignited, it gives an inorganic residue containing sodium, potassium, and phosphate. Lead acetate and barium chloride solutions precipitate the phospholipid from its aqueous emulsion.

Analytical values were: C, 53·0; H, 8·5; N, 1·26; P, 5·2; Ac, 11·4%. Equimolecular proportions of palmitic and oleic acids (the known major constituent acids) being assumed, formula (II), $C_{61}H_{113}O_{29}NP_2$, requires C, 52·8; H, 8·2; N, 1·01; P, 4·6; Ac, 14·7%. High accuracy cannot be expected in view of the above assumption, and also because of the small unknown content of sodium and potassium. The most significant feature is the N–P ratio of 1 : 1·85, in marked contrast to those (1 : 1 and 2 : 1) hitherto found for phosphatides. The acetyl value indicates that there has been some loss of sugar, possibly in the technical extraction of the phosphatide.

Bases Present.—The only basic substances found were ethanolamine (R_F 0·53) and ethanolamine phosphate (R_F 0·2). At the time, the latter had not been identified among the hydrolysis products of phospholipids and a few mg. were extracted with boiling water from the appropriate section of the chromatogram paper and compared with synthetic ethanolamine phosphate (obtained in 48% yield by Outhouse's method, *Biochem. J.*, 1936, **30**, 197). Chromatographic

comparison with ninhydrin and phosphate sprays showed identity, and the m. p.s of the flavianates of synthetic, natural, and mixed products were 225°, 224°, and 224°, respectively.

Identification of Other Hydrolysis Products.—(*a*) *Glycerol and inositol phosphates.* For paper partition (Hanes and Isherwood, *Nature*, 1949, **164**, 1107), basic acetate–pyridine–water was the most suitable mixture [water-poor phase from ethyl acetate (100 c.c.), pyridine (45 c.c.), and water (100 c.c.)]. The glycerinositophosphatide (0·800 g.) was emulsified with N-sulphuric acid (50 c.c.) and heated for 6 hr. on a boiling-water bath. The insoluble precipitate of fatty acids, which formed overnight, was filtered off and the filtrate was neutralised with barium hydroxide solution (phenolphthalein). Insoluble barium salts were filtered off and the filtrate was treated with sufficient ammonium sulphate to precipitate the remaining barium. After filtration, water was distilled off *in vacuo* and the resulting syrupy liquid which contained ammonium salts of phosphoric esters, was chromatographed (see above) for 36 hr. at 18°. The dried paper was sprayed with the perchloric acid–ammonium molybdate spray (Hanes *et al.*). In addition to an indistinct spot near the starting point, probably due to traces of the slow-moving ethanolamine phosphate, there appeared three more distinct spots of R_F 0·25, 0·68, and 0·75, which were in agreement with values found for authentic ammonium salts of inositol phosphate (or mixture of phosphates) and α- and β-glycerophosphate, respectively.

More drastic hydrolysis yielded glycerol and inositol: The glycerinositophosphatide (100 mg.) was heated in a sealed tube with 3N-sulphuric acid (2 c.c.) for 4 hr. at 160—170°. On cooling, the precipitated fatty acids were filtered off and the filtrate was neutralised with barium carbonate. After filtration from the barium salts, the filtrate was concentrated *in vacuo* to a syrup. This was spotted, together with glycerol and inositol, on a paper chromatogram which was run for 12 hr. (butanol–water), and then sprayed with ammoniacal silver nitrate. The syrup yielded two spots of R_F 0·03 and 0·33, which agreed with those of inositol and glycerol, respectively.

Isolation of phosphoric esters. The glycerinositophosphatide (10 g.) was refluxed for 6 hr. with concentrated sulphuric acid (2·5 g.) in methanol (100 c.c.). After cooling, the methanol was removed *in vacuo* and the residue was extracted with water (50 c.c.). Insoluble material was filtered off and the filtrate was extracted with light petroleum (b. p. 40—60°) to remove traces of fatty material, concentrated *in vacuo* to *ca.* 20 c.c., and neutralised with barium hydroxide solution (thymolphthalein). Insoluble barium salts were filtered off, and the filtrate was concentrated by distillation under reduced pressure in a carbon dioxide-free atmosphere. Trituration of the syrupy residue with ethanol precipitated the barium salts of the phosphate esters, which were filtered off, washed with ethanol, and dried (yield, 5 g.). Should the product at this stage give a slight Molisch test, traces of carbohydrates are removed by Soxhlet extraction with 90% ethanol.

The barium salts were dissolved in distilled water (20 c.c.), and the barium was removed by exact neutralisation with dilute sulphuric acid and filtration. The filtrate was concentrated to dryness *in vacuo* and the resulting syrup triturated with ethanol (10 c.c.) which caused precipitation of a solid (A) which was filtered off (see below).

Isolation of barium glycerophosphate. The above filtrate was concentrated *in vacuo*; a small amount of solid which separated was filtered off. After complete removal of the ethanol, the resulting syrup was dissolved in water (2 c.c.) and neutralised with a saturated solution of barium hydroxide (thymolphthalein) and poured into twice its volume of ethanol. After being kept overnight in the refrigerator the crystalline precipitate which had separated was filtered off, washed with ethanol, and dried (P_2O_5) *in vacuo* (yield, 1·9 g.) (Found: C, 12·0; H, 2·1; P, 9·6. Calc. for $C_3H_7O_6PBa$: C, 11·7; H, 2·3; P, 10·1%).

This barium salt (1 g.) was hydrolysed with 3N-sulphuric acid (5 c.c.) at 160—170° in a sealed tube for 3 hr. After removal of the sulphuric acid (barium carbonate) and concentration *in vacuo*, the resulting syrup gave a single spot corresponding to glycerol when submitted to chromatographic analysis. The remainder of the syrup (0·25 g.), when benzoylated in pyridine, gave a 90% yield of glycerol tribenzoate, m. p. and mixed m. p. 76° (from light petroleum).

Inositol dihydrogen monophosphate. Solid A (above), when dried *in vacuo* (P_2O_5), was a white crystalline, slightly hygroscopic solid (1·8 g.), acid to litmus and Congo-red, and responding to the Scherer test (Found, in a sample dried over P_2O_5 at 118° *in vacuo*: C, 27·3; H, 5·1; P, 11·55. Calc. for $C_6H_{13}O_9P$: C, 27·7; H, 5·0; P, 11·9%). Two equivalent weights were found, *viz.*, 257·8 (screened methyl-red) and 128·3 (thymolphthalein) (calc.: 260, 130).

Barium inositol monophosphate. The above acid (1 g.), dissolved in water (1 c.c.), was neutralised (thymolphthalein) with saturated barium hydroxide solution. A faint precipitate was removed by filtration and the filtrate was concentrated to *ca.* 5 c.c. and poured into ethanol

(10 c.c.). The resulting precipitate (1·5 g.) was filtered off, washed with ethanol and dried over P_2O_5 *in vacuo* at 118° (Found: C, 18·1; H, 3·0; P, 7·85. Calc. for $C_6H_{11}O_9PBa$: C, 18·2; H, 2·8; P, 7·8%). This barium salt (20 mg.) was converted into the ammonium salt for chromatographic analysis as described earlier, and analysed for phosphate (ethyl acetate–pyridine–water). One spot only was observed, of R_F 0·24, compared with 0·25 found for the ammonium salt of one of the phosphate esters produced on hydrolysis.

Isolation of inositol. The above barium salt (1·4 g.) was hydrolysed by heating with 3N-sulphuric acid (5 c.c.) at 160—170° in a sealed tube for 3 hr. After cooling, the mixture was neutralised with barium carbonate and the insoluble barium salts were filtered off. The filtrate was taken to dryness *in vacuo*, and the solid residue was triturated with ethanol. After filtration, washing with ethanol, and drying, there remained 0·53 g. of crystalline material. Crystallisation from water yielded 0·47 g. of optically inactive crystalline material, m. p. 220°; the mixed m. p. with inactive inositol was 222°. The identity was also confirmed through the hexa-acetate, m. p. 218°.

Identification and isolation of sugars. The glycerinositophosphatide (50 mg.) was hydrolysed with 0·5N-sulphuric acid (2 c.c.) in a sealed tube at 100° for 12 hr. After cooling, fatty acids were removed by filtration, and the filtrate was neutralised with barium carbonate, filtered, and concentrated to a thin syrup by distillation under reduced pressure. Spots of this syrup and a standard mixture containing galactose, arabinose, ribose, and rhamnose were placed on a paper chromatogram, which was run for 24 hr. (*n*-butanol saturated with water as mobile phase). The paper was dried, sprayed with ammoniacal silver nitrate, and heated in front of an electric fire. Two spots appeared corresponding closely in R_F to galactose and arabinose, but as mannose and fructose have R_G close to that of arabinose, the presence of the latter was confirmed by a similar analysis of a mixture containing galactose, arabinose, fructose, and mannose (spraying with a 1 : 1 mixture of 5% aqueous trichloroacetic acid and 1% alcoholic aniline, which gives a rose-red spot with pentoses, and brown spot with hexoses; cf. Hough, Jones, and Wadman, *J.*, 1950, 1702).

Weaker acid hydrolysis. In order to determine the nature of the original saccharide present, the glycerinositophosphatide (50 mg.) was refluxed for 12 hr. with 0·01N-sulphuric acid (5 c.c.). The mixture was cooled and filtered through a "Filtercel" pad. After neutralisation with barium carbonate, and removal of barium salts by filtration, the filtrate was evaporated *in vacuo* to a thin syrup and submitted to chromatographic analysis, as above. Silver nitrate showed the presence of a new slower-moving sugar and arabinose, and the aniline–trichloroacetic acid mixture gave a brown spot for the slow-moving sugar and confirmed the presence of arabinose by a rose-pink spot.

On further hydrolysis of the syrup with 0·5N-sulphuric acid in a sealed tube at 100° for 12 hr., neutralisation, concentration, and chromatographic analysis, as above, gave a syrup, containing galactose and arabinose, the latter being present in larger amount than in the previous experiment. It appeared therefore that the slow-moving sugar was probably a disaccharide containing galactose and arabinose. In order to confirm this, a broad chromatogram was started with a large number of drops of the syrup. The position of the slow-moving sugar was found by developing a thin strip, and the remainder of the chromatogram containing this spot was cut out and extracted with water. The extract was evaporated to a syrup and hydrolysed by 0·5N-sulphuric acid (1 c.c.) in a sealed tube for 12 hr. at 100°; after neutralisation and concentration as above described, chromatographic analysis showed the presence of both galactose and arabinose.

Molecular ratio of sugars present. The method used for the quantitative estimation of the sugars was that described by Hirst and Jones (*J.*, 1949, 1659) (cf. also Flood, Hirst, and Jones, *J.*, 1948, 1679), based on paper-chromatographic separation of the sugars and subsequent determination by titration of the formic acid liberated on oxidation with sodium periodate. The glycerinositophosphatide (100 mg.) was hydrolysed with 0·5N-sulphuric acid, as described above under the identification of sugars, and the sugars were obtained as a thin syrup. Uniform drops of the latter were placed at intervals of 0·5 cm. on the starting line of a Whatman No. 1 filter-paper sheet, by means of a hypodermic syringe fitted with a micrometer screwhead. In order to obtain uniform bands on the chromatogram, it is important that the drops should be of the same volume, and that they should not be allowed to coalesce. The chromatogram was then run for 54 hr. (butanol–water) in a covered tank well protected from draught. After drying, thin strips were cut from both edges and from the centre of the filter paper sheet, and developed in order to locate the positions of the sugar zones. These were then cut out from the undeveloped chromatogram, and extracted with hot water, by suspension from the bottom

of a coil reflux condenser (see Flood *et al.*, *loc. cit.*, p. 1681, for details). To each extract (*ca.* 5 c.c.) 0·25M-sodium periodate (0·5 c.c.) was added, and the solution was heated under a reflux for 20 min. After cooling, excess of periodate was destroyed by ethylene glycol (0·5 c.c.), and the solution was titrated against 0·01N-sodium hydroxide (light-screened methyl-red). Blanks were carried out simultaneously on portions of the chromatogram which contained no sugar. The following values were obtained: galactose required 0·94 c.c. of 0·01N-NaOH, equiv. to 0·338 mg. of galactose; * arabinose required 1·74 c.c. of 0·01N-NaOH, equiv. to 0·653 mg. of arabinose.* Mol. ratio, galactose : arabinose = 1 : 2·3.

Isolation and characterisation of L-*arabinose and* D-*galactose.* The sugars obtained by acid hydrolysis were separated on a column of powdered cellulose (*n*-butanol–water), with the automatic receiver changer described by Hough, Jones, and Wadman, *J.*, 1949, 2511.

Acid Hydrolysis of the Glycerinositophosphatide (*X*).—The phosphatide (5 g.) was refluxed for 8 hr. with 4% methanolic hydrogen chloride (20 c.c.). After cooling and filtration, the solvent was removed from the filtrate *in vacuo*, leaving a viscous syrup. This was dissolved in water (1 c.c.) and neutralised by passage down a small glass column packed with Amberlite IR-4B. The resulting solution of methyl glycosides was concentrated *in vacuo* to a syrup. This was hydrolysed with N-sulphuric acid (2 c.c.) in a sealed tube at 100° for 2 hr. The hydrolysate was neutralised ($BaCO_3$), filtered, and concentrated to dryness by distillation of solvent *in vacuo*. A paper chromatogram of the resulting syrup showed the presence of galactose and arabinose. The remainder of the syrup, dissolved in *n*-butanol (1 c.c.), was placed on top of a cellulose column and washed on to the column with several 1-c.c. portions of *n*-butanol–water. The constant-head reservoir which contained this solvent mixture was then placed in position and run for 3 days, after which it was placed over the constant receiver-changer. Two chromatographically pure fractions were obtained, and these were evaporated to dryness *in vacuo* and extracted with ether in order to remove traces of oily impurity. Each fraction was dissolved in water (2 c.c.), filtered through a sintered-glass funnel, and concentrated *in vacuo* (over P_2O_5), whereupon the sugars crystallised. They were triturated with ethanol, filtered, and dried, yielding fractions 1 (31·1 mg.) and 2 (15·3 mg.).

Fraction 1. $[\alpha]_D^{20}$ +108° (equilibrium) and R_G 0·12 agree with those of authentic L-arabinose. 20 mg. yielded 32 mg. of toluene-*p*-sulphonylhydrazone, m. p. and mixed m. p. 154°. 10 mg. yielded 19·6 mg. of arabinosazone, m. p. and mixed m. p. 165°.

Fraction 2. $[\alpha]_D^{20}$ +82·9° (equilibrium) and R_G 0·07 agreed with those of D-galactose. 8 mg. yielded 13·8 mg. of an osazone with the characteristic appearance of galactosazone under the microscope, and m. p. 196° (mixed m. p. 195°). 6 mg., oxidised with nitric acid, yielded 6 mg. of an acid, m. p. 211°; the mixed m. p. with mucic acid (213°) was 212°.

Alkaline Hydrolysis of Glycerinositophosphatide (*X*).—The phosphatide (40 g.) was dissolved in moist benzene (400 c.c.) and to the cold solution was added a solution of potassium hydroxide (9·6 g.) in methanol (120 c.c.). A gelatinous precipitate separated after about 10 min. After 20 hr., with occasional shaking, at room temperature, the precipitate was filtered off at the pump and washed with several changes of 3 : 1 benzene–methanol. The filtrate and washings, which contained soaps and fatty methyl esters, were retained.

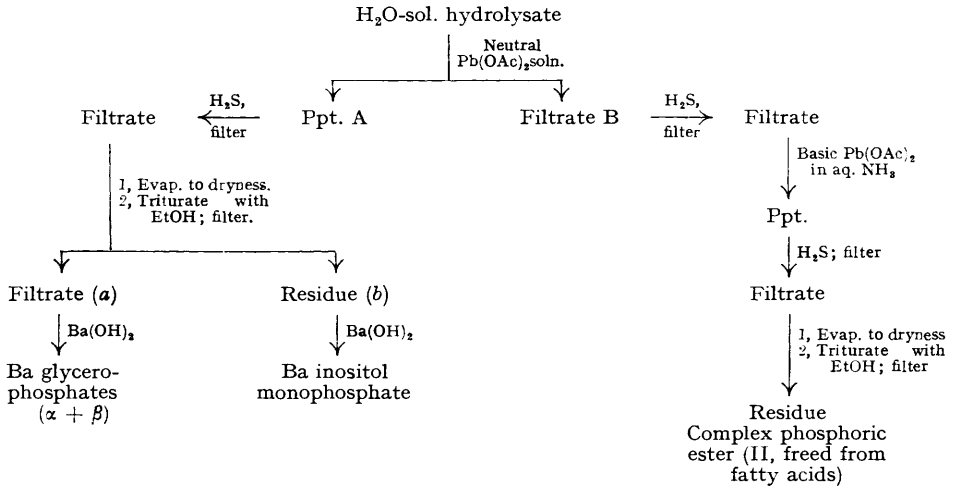
The precipitate was extracted with water (200 c.c.), and the cloudy solution acidified with acetic acid and extracted with light petroleum (b. p. 40—60°). The aqueous layer was clarified by filtration through a No. 3 sintered-glass funnel with the aid of a "Filtercel" pad. Excess of acetic acid was removed from the filtrate by several extractions with ether, and the solution was concentrated to about 80 c.c. The phosphoric esters present were separated according to the scheme on page 3477.

Neutral lead acetate precipitation. A slight excess of a saturated solution of neutral lead acetate was added to the above aqueous hydrolysate and the mixture was shaken vigorously from time to time and kept overnight. The lead salts (precipitate A in scheme) were then filtered off ("Filtercel" pad), washed well with distilled water, suspended in distilled water (20 c.c.) and treated as shown in the scheme. The filtrate (*a*) and washings were concentrated to dryness *in vacuo* and the residue was dissolved in water (2 c.c.). The strongly acid solution was neutralised with aqueous barium hydroxide and poured into twice its volume of ethanol. The resulting precipitate was filtered off and dried *in vacuo* (P_2O_5) (yield, 90·6 mg.). By the methods already described it was shown to be a mixture of barium salts of α- and β-glycero-

* Calc. on the basis of 100% conversion into formic acid: conversion is nearer 95 ± 3%, but this does not greatly affect the mol. ratio of the sugars. The ratio found supports the view expressed earlier that some of the phosphatide has lost part of the sugar fragment, probably in the form of a galactose arabinose disaccharide.

phosphates. The residue (*b*), similarly treated, gave a precipitate (98·3 mg.) of the barium salt of inositol phosphate.

The filtrate B and washings were freed from lead and concentrated *in vacuo* to 20 c.c. This solution was neutral to Congo-red, but acid to litmus, and gave a strong positive test for phosphate, and hence, presumably, contained a phosphate ester. This was precipitated by the



addition of slight excess of a saturated solution of basic lead acetate in 4% aqueous ammonia. The white lead salt was filtered through a No. 3 sintered glass funnel, well washed with distilled water, suspended in distilled water (300 c.c.), and treated as shown in the scheme. The final crystalline residue (dried *in vacuo* at room temperature over P_2O_5) was triturated with ethanol, filtered off at the pump, and well washed with ethanol. It was dried at room temperature in a high vacuum (P_2O_5) (yield, 3·1 g.).

The resulting complex *ester* (Y) is a white, crystalline, extremely hygroscopic solid (Found : C, 36·5; H, 5·8; N, 1·46; P, 6·8. $C_{27}H_{51}O_{27}NP_2$ requires C, 36·7; H, 5·8; N, 1·58; P, 7·0%). It contains nitrogen and phosphorus, and gives positive tests for carbohydrates (Molisch), inositol (Scherer), and glycerol. It does not reduce Fehling's solution or give the ninhydrin test until after hydrolysis with dilute mineral acid.

Approx. 150 mg. of the phosphate were dried at 61° (P_2O_5) in a high vacuum, weighed, and dissolved in distilled water (to give 50 c.c.). Portions (10 c.c.) were titrated against 0·01N-sodium hydroxide (phenolphthalein) [Found : Equiv., 445·4. (II) requires Equiv., 441·5]. When light-screened methyl-red was used as indicator, only one drop of the standard alkali was required for neutralisation *i.e.*, there is no strong acid equivalent, and both phosphate groups are in the same state of weak acidity.

Chromatograms of the Complex Phosphate (*Y*).—This ester was subjected to paper chromatography, with the following solvent mixtures and spraying with both sugar and phosphate spray reagents : (1) *n*-butanol saturated with water; (2) *n*-butanol saturated with water–morpholine (3 : 1); (3) pyridine–ethyl acetate–water [water-poor phase from pyridine (45 c.c.), ethyl acetate (100 c.c.), and water (100 c.c.)]. Each paper was run for 60 hr., and one half was sprayed with ammoniacal silver nitrate and the other with perchloric acid–molybdate reagent. All the papers showed one distinct spot only, independent of which spray was used, and it was concluded that the compound is a single homogeneous substance.

Acid Hydrolysis of the Glycerinositophosphate (*Y*).—(1) *To galactose and arabinose.* The ester (20 mg.) was hydrolysed in a sealed tube at 100° for 12 hr. with 0·5N-sulphuric acid (1 c.c.). After being freed from sulphuric acid ($BaCO_3$) the neutral filtrate was concentrated *in vacuo* to a syrup which on chromatographic analysis as described earlier gave two spots corresponding in R_G and colour to galactose and arabinose.

(2) *Bases.* The ester (30 mg.) was hydrolysed by refluxing with 6N-hydrochloric acid (3 c.c.) for 12 hr. After evaporation to dryness *in vacuo* (over KOH), the residue was dissolved in water (1 c.c.), neutralised with sodium hydroxide solution, and submitted to paper chromatographic analysis together with a mixture containing 2-aminoethyl phosphate and ethanolamine

hydrochloride. A 3 : 1 mixture of *n*-butanol saturated with water and morpholine was used as the mobile phase. The paper was sprayed with ninhydrin and showed the presence of ethanolamine and 2-aminoethyl phosphate.

(3) *To inositol and glycerol.* The ester (20 mg.) was hydrolysed with 3N-sulphuric acid (1 c.c.) at 160° in a sealed tube for 3 hr. After neutralisation ($BaCO_3$), filtration, and concentration *in vacuo*, paper-partition chromatography (*n*-butanol–water) showed the presence of inactive inositol and glycerol.

(4) *The phosphoric acids.* The ester (10 mg.) was hydrolysed by boiling 2N-sulphuric acid (2 c.c.) for 6 hr. The hydrolysate was freed from sulphuric acid (barium hydroxide), and the soluble barium salts were converted into ammonium salts as described earlier (p. 3474), and submitted to chromatographic analysis (ethyl acetate–pyridine–water). Three spots were observed, corresponding to salts of inositol monophosphoric acid and α- and β-glycerophosphoric acid.

Isolation of barium glycerophosphate and barium inositol monophosphate. The ester (Y) (150 mg.) was hydrolysed with 2N-sulphuric acid (5 c.c.) at 100° for 6 hr. The solution was cooled and neutralised with barium hydroxide solution (thymolphthalein), and after filtration was evaporated to dryness under reduced pressure. The residue was triturated with ethanol, and the insoluble barium salts were filtered off and dried (yield, 112 mg.). The barium salts were dissolved in a few c.c. of water, and the barium was quantitatively removed (H_2SO_4). The filtrate was evaporated to dryness under reduced pressure and the residue triturated with ethanol and filtered. After removal of ethanol from the filtrate under reduced pressure, the residue was dissolved in a few c.c. of water, neutralised (barium hydroxide), poured into twice its volume of ethanol, and kept overnight in the refrigerator. The precipitated barium salt was filtered and dried *in vacuo* at 118° (P_2O_5) (yield, 45·3 mg.). The salt was shown, by chromatography and further hydrolysis to glycerol, to be a mixture of barium salts of α- and β-glycerophosphoric acid. The residue from the above trituration was dissolved in a few c.c. of water, neutralised (barium hydroxide), concentrated to a small volume, and poured into twice its volume of ethanol. The precipitated barium salt was filtered off and dried at 118° (P_2O_5) (yield, 60·9 mg.). This was shown by chromatography and further hydrolysis to inositol to be barium inositol monophosphate.

The molecular ratio of the glycerol and inositol phosphate found was 1 : 1·04.

Quantitative estimation of sugars. This was carried out by the periodate method described above. The glycerinositophosphate (60 mg.) was hydrolysed with 0·5N-sulphuric acid (2 c.c.) in a sealed tube at 100 for 12 hr. The galactose fraction required 0·81 c.c. of 0·01N-NaOH, equiv. to 0·2919 mg. of galactose, and the arabinose fraction required 1·23 c.c., equiv. to 0·4616 mg. of arabinose, being a molecular ratio, galactose–arabinose, 1 : 1·89.

Fatty Acid Content of Glycerinositophosphatide (Y).—Insofar as our results go beyond those of Hilditch and Zaky (*loc. cit.*), they are in agreement with theirs that the main acids present were oleic 47, linoleic 23, and palmitic 17%.

The retained filtrate and washings from the alkaline hydrolysis of the phosphatide were freed from solvent under reduced pressure and the residue was extracted with acetone and filtered. The filtrate contained methyl fatty esters (A), and the residue consisted of potassium soaps (B). Distillation of A yielded an oil (11·7 g.), b. p. *ca.* 147°/0·0084 mm. (I val., 86·9). Hydrogenation converted the oil into a white, crystalline solid (from methanol at −20°), m. p. 31—33°, which on saponification yielded an acid mixture, m. p. 67°, set point, 63°. The long *X*-ray spacing, m. p., and analysis agreed with those for a stearic-rich mixture of palmitic and stearic acids. The potassium salts, B, yielded acids (5·4 g.) (I val., 7·3). After hydrogenation and crystallisation from methanol, they had m. p. 66° and set point 62—64°, the *X*-ray spacing agreeing with that for a mixture of palmitic and stearic acids. The total amount of acid obtained was 16 g. (40% of the wt. of the phosphatide).

The above acids were obtained after somewhat mild hydrolysis at room temperature, but hydrolysis of a methanolic-benzene solution with boiling methanolic potassium hydroxide yielded much the same amount of fatty acid, which on no occasion exceeded 40·4%

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