PLANT PHOSPHOLIPIDS

I. IDENTIFICATION OF THE PHOSPHATIDYL GLYCEROLS*

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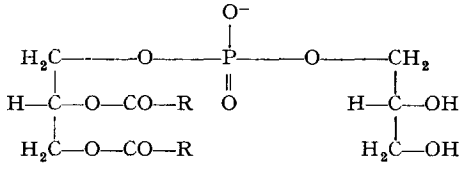
The identification of α, α'-diglycerophosphate in *Scenedesmus*[1,2] and of cytidine diphosphate glycerol[3] in *Lactobacillus arabinosus* suggested the possible existence of the phosphatidyl glycerols. These compounds could be formed by a reaction exactly analogous to that demonstrated by KENNEDY AND WEISS[4] for the biosynthesis of the lecithins and would possess the structure



A major point of confusion in the phospholipid literature rests with the difficulties inherent in identification of partially purified lipids which have little or no nitrogen. The existence of the phosphatidic acids in nature was first questioned by HANAHAN AND CHAIKOFF[5] and their contention has recently been verified by KATES[20]. MARINETTI reported absence of phosphatidic acids in tissues. That there has existed an important non-nitrogenous component besides the inositol lipids is almost clear from the remarks of a host of workers and reviewers.

Small amounts of phospholipids containing polyglycerophosphate have been reported by PANGBORN[7] (cardiolipin), FLEURY[8] (glycérophosphatogène), MCKIBBIN AND TAYLOR[9] (polyglycerolphosphatide) and IGARASHI *et al.*[10]. PANGBORN had considered the possibility of a bis-glycerophosphatidic acid structure for cardiolipin. Interest in the properties of these lipids led BAER[11] to prepare several enantiomeric α-bisphosphatidic acids.

The existence of the phosphatidyl glycerols appears to have been overlooked because the simplicity of their structure and the identity of their degradation products with those of other glycerolphosphatides produced no profound changes in phospholipid analytical results. A correlation of the properties of fractionated lipids and the nature of the glycerophosphoryl esters resulting from their degradation has led to the identification of this group of glycerolphosphatides.

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MATERIALS AND METHODS

Lipids of Scenedesmus-^{32}P

Scenedesmus cells (0.5 g) were allowed to grow 24–72 hours at 1000 ft.-c. in 50 ml of culture medium containing no phosphorus other than that in 3 mc (0.1 mg) radiophosphate. The cells were washed with water and four volumes of hot absolute alcohol was added to the packed cells. The 80% alcohol extract was chromatographed two-dimensionally on unwashed Whatman No. 1 or No. 4 paper using phenol–water and butanol–propionic acid–water solvents[12]. The ^{32}P-lipid components separated into four distinct spots the two fastest possessing the greatest radioactivity (Fig. 1, Table I).

Phospholipids-^{32}P of leaves

Excised leaves of tobacco, barley, sweet clover (*Melilotus alba*) and of white clover (*Trifolium repens*) were illuminated for 15 hours with their stems in a neutral solution of radiophosphate (1 mc/ml). The stems were rinsed with water and the leaf plunged into hot 95% ethanol. Further extracts with 80% and 100% ethanol were combined and concentrated to 0.2 ml. Aliquot samples were chromatographed on Whatman No. 4 paper. The lipid areas were eluted with pyridine and evaporated to dryness at room temperature.

Deacylation of ^{32}P-lipids

After removal of the pyridine the lipids were taken up in 50 μl ethanol containing 5% carbon tetrachloride. To this solution was added 100 μl of 0.2 N KOH in methanol[13]. After 20 minutes or 5 hours at 37° an equivalent amount of dry Dowex-50-H^+ was added. Addition of a drop of water and ten seconds stirring gave a neutral solution suitable for chromatography. The cation exchange resin which was washed with ethanol to remove residual lipids or products did not adsorb appreciable amounts of GPE or GPC*.

Chromatography of the glyceryl phosphoryl esters

Using the same solvents systems with unwashed filter papers the products of hydrolysis were easily separated with R_F values given in Table I. All of the lipid ^{32}P was found in these compounds. No detectable orthophosphate or glycerophosphate was formed. Phospholipid analyses were performed by direct counting of hydrolysate chromatograms such as that for tobacco leaf lipids shown in Fig. 4.

Lead tetraacetate oxidation of phosphatidyl glycerols

To the eluate of ^{32}P-lipid II in acetic acid solution was added 2 mg of freshly prepared lead tetraacetate and an equivalent amount of ethanol. After one hour an equivalent amount of ethylene glycol was added. Water was added and the lipid material taken up in chloroform. The chloroform solution was evaporated to dryness and the residue product hydrolyzed as before. Chromatography of the deacylated product gave approximately equal amounts of GP and a product with considerably higher R_F in phenol–water and equal R_F in butanol–propionic acid–water than that of GPG.

Benzoylation of phosphatidyl glycerols

A sample of the eluted phosphatidyl glycerols was tested for free hydroxyl groups by benzoylation with benzoyl chloride in pyridine solution. After two minutes at forty degrees the reaction mixture was diluted with water and extracted with petroleum ether. After evaporation of the solvent, 100 μl of 0.2 N KOH in methanol was added and allowed to stand 30 minutes at 37°. Chromatography of the decationized product revealed a major component with $R_F = 0.93$ in phenol-water and $R_F = 0.92$ in butanol–propionic acid–water which was neither the original lipid nor GPG and had the mobility anticipated for a dibenzoyl ester of GPG. A second component of the hydrolysate contained 30% of the total ^{32}P and had an R_F in phenol of 0.70 which suggested its identity as γ-benzoyl-α, α'-diglycerophosphate. Each benzoylated product was hydrolyzed separately in 1.0 M methanolic potassium hydroxide at 70° for one hour. The solutions were decationized with excess Dowex-50-H^+ and chromatographed in phenol–water. For each hydrolysis the products were found to be 70% GPG and 30% GP.

Acetonation of phosphatidyl glycerols

A sample of the phosphatidyl glycerols was tested for vicinal hydroxyl groups by reaction with dry acetone in the presence of hydrogen chloride. The labeled lipid in 0.5 ml acetone containing 1% HCl was allowed to stand two days at room temperature. The sample was then allowed to evaporate

* The following abbreviations will be used in this article: CTP, cytidine triphosphate. CMP, cytidine monophosphate. CDPGlycerol, cytidine diphosphate glycerol. GPG, glycerophosphorylglycerol. GPC, glycerophosphorylcholine. GPE, glycerophosphorylethanolamine. GPInos, glycerophosphorylinositol. GP, L-α-glycerophosphate = D-glycerol-1-phosphate. POP, pyrophosphate.

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slowly in a desiccator over KOH. The residue was taken up in ethanol (0.05 ml) and treated with 0.1 ml of 0.2 *N* methanolic KOH for 30 minutes at 37°. Without acidification an aliquot portion of the solution was chromatographed in phenol–water solvent on Whatman No. 4 paper. The new radioactive product, $R_F = 0.89$ was observed. This product was eluted and treated with Dowex-50-H^+ at 60° for ten minutes. Cochromatography in phenol–water demonstrated the identity of the hydrolysis product with GPG.

RESULTS AND DISCUSSION

Chromatography of *Scenedesmus*-^{32}P extracts separated the phospholipids into four distinct groups shown in Fig. 1. Lipids I and II do not separate in phenol but can be resolved in the butanol-propionic acid-water solvent. Approximate R_F values and total ^{32}P activities of these groups are given in Table I. Refined techniques[14] may separate these more completely and reveal minor components. Extracts of *Melilotus alba* and of *Trifolium repens*, two species of clover, gave similar results but with only the three major P-lipid areas. The phosphatidyl ethanolamines and the phosphatidyl serines occurred in smaller amounts and were estimated by determining the ^{32}P activity in glycerophosphorylethanolamine and glycerophosphorylserine obtained on hydrolysis of eluates of the total lipid areas from chromatograms.

TABLE I

R_F VALUES OF *Scenedesmus* PHOSPHOLIPIDS AND THEIR HYDROLYSIS PRODUCTS

	Phospholipids			*KOH Hydrolysate*		
	% Total	*R_F Phenol**	*R_F Bu-Pr*	*R_F Phenol*	*R_F Bu-Pr*	*Identified as*
I	36.1	0.90	0.94	0.89	0.29	GPC
II	41.2	0.87	0.85	0.40	0.17	GPG
III	14.6	0.73	0.68	0.12	0.05	GPInos
IV	9.0	0.57	0.59			(GPInos)**
	1.0			0.22	0.12	GPSer***
	6.1			0.65	0.23	GPE***

* R_F values for *Scenedesmus* phospholipids on Whatman No. 4 unwashed paper and calculated for the center of density of the spots. Chromatograms were descending type developed with (1) 75% phenol and (2) butanol–propionic acid–water.

** Lipid IV was only 10% hydrolyzed. The product was GPInos.

*** Determined from hydrolysate of total lipids.

The lipids I, II and III were classified according to their products of deacylation which showed them to be surprisingly homogeneous (Fig. 2, Fig. 3). R_F values for these products are given in Table I. Minor hydrolysis products could be accounted for by contamination with material from adjacent spots. The composition of phospholipids of *Scenedesmus*-^{32}P cells and of leaves of tobacco^{32}P, barley-^{32}P and clover-^{32}P are given in Table II.

It is seen that the lecithins have the highest R_F and concentrations and are followed in both respects by the phosphatidyl glycerols. This observation immediately precluded the existence of large amounts of α-bisphosphatidic acids which would have higher R_F's than either the lecithins or the phosphatidyl glycerols. Further experiments to determine the structure were performed. The phosphatidyl glycerols were oxidized with lead tetraacetate and hydrolyzed to give a product, glycerophos-

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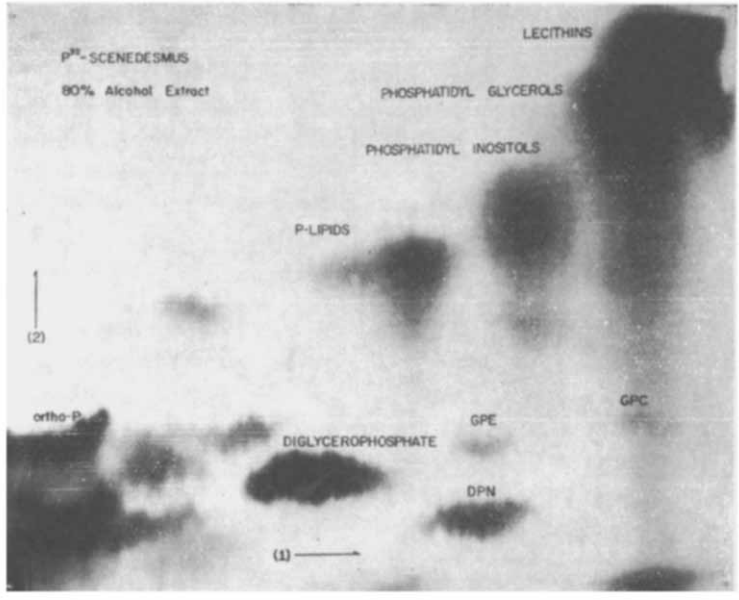

Fig. 1. Radiogram of alcohol extract of *Scenedesmus* labeled with radiophosphate. Chromatogram was developed in direction (1) with phenol–water and in direction (2) with butanol–propionic acid water.

TABLE II

COMPOSITION OF PLANT PHOSPHOLIPIDS

Phospholipid	*Scenedesmus* %	*Tobacco* %	*Sweet clover* %	*Barley* %
Lecithin	36.1	46.5	30.0	52.4
Phosphatidyl glycerol	41.2	22.0	24 9	22.6
Phosphatidyl inositol	14.6	22.4	17.5	14.0
Phosphatidyl ethanolamine	6.1	7.9	8.6	1.4
Phosphatidyl serine	1.0	0.7	6.3	—

phorylglycolaldehyde which was shown to be different from GPG by failure in cochromatography. The phosphatidyl glycerols were benzoylated and then deacylated to give a benzoate of diglycerophosphate. An acetone derivative of the phosphatidyl glycerols was prepared and deacylated to give a new compound with chromatographic properties anticipated for those of monoisopropylidene-α,α'-diglycerophosphate. These observations indicate that the naturally occurring lipids are phosphatidyl glycerols rather than α-bisphosphatidic acids.

Hydrolysis of P-lipid III from each of the plants examined gave pure glycerophosphorylinositol the structure of which is not yet rigorously proved. Its acid hydrolysis products provide evidence for its structure as named. It was demonstrated by TODD AND BROWN[15] that α-hydroxy diesters of phosphoric acid hydrolyze by cyclization to an intermediate 1,2-cyclic phosphate followed by cleavage. By such a process one would expect glycerophosphorylinositol to yield more than twice as much inositol phosphate than α-glycerophosphate since the two α-hydroxyl groups of inositol are forced closer to the phosphorus atom than is the α-hydroxyl group of glycerol. After

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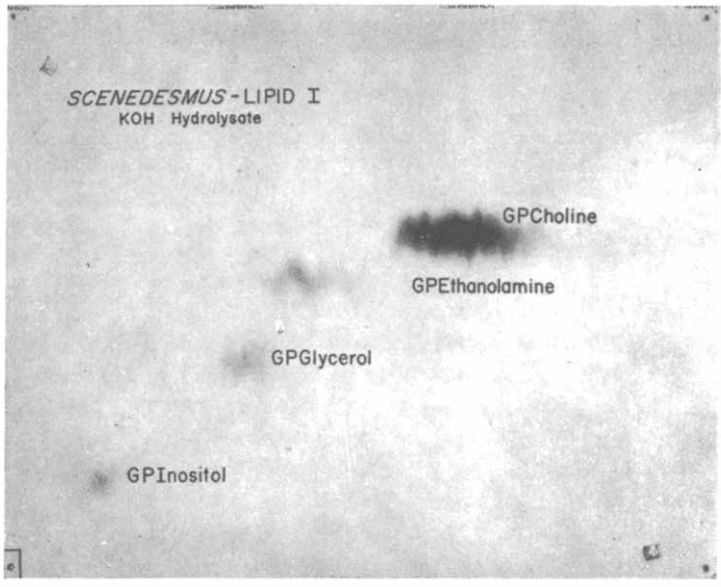

Fig. 2. Methanolic KOH hydrolysate of Lipid I.

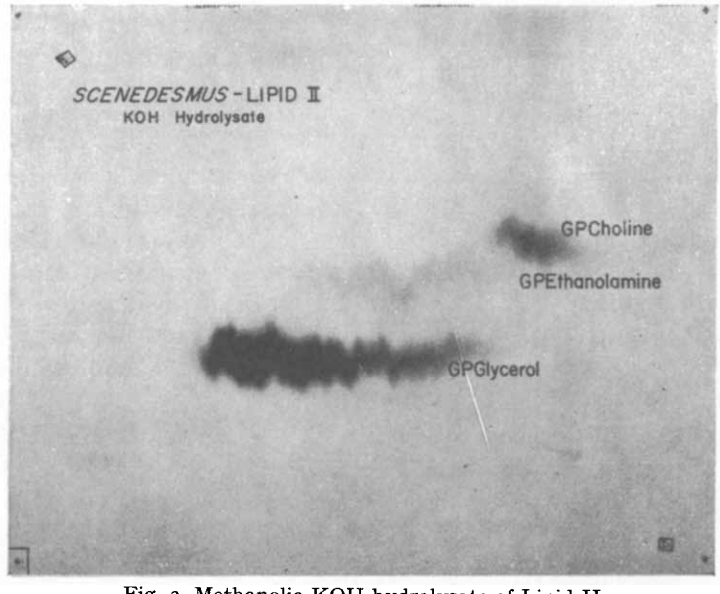

Fig. 3. Methanolic KOH hydrolysate of Lipid II.

ten minutes at 100° in 1 *N* hydrochloric acid the hydrolysate of this ester was chromatographed and the two products counted. The ratio of inositol phosphate to glycerophosphate was 2.95:1. These observations are in accord with the α-phosphatidyl inositol structure proposed by FAURE AND MORELEC-COULON[16] for a wheat germ lipid and with the results reported by WAGENKNECHT AND CARTER[17] for a phosphatidyl inositol of peas.

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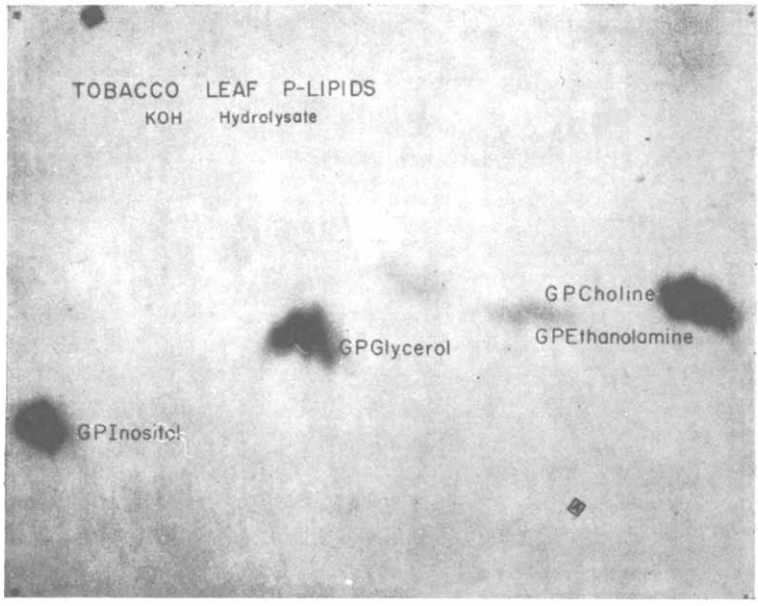

Fig. 4. Methanolic KOH hydrolysate of total phospholipids of tobacco leaf.

The amount of glycerophosphate formed during any of these hydrolyses was scarcely detectable. It is clear that phosphatidic acids do not exist in the tissues studied so far. Further it is now even more apparent that a major fraction of phosphatidic acids previously reported arise as postulated by HANAHAN AND CHAIKOFF[5] as a result of action of phosphatidase C or by action of mineral acid during purification procedures. The acid hydrolysis of GPG is very rapid. The half time in 1.0 N HNO_3 at 100° was found to be close to two minutes[2]. Evaporation of hydrochloric acid solutions of GPG at paper chromatogram origins resulted in extensive hydrolysis. This treatment presumably corresponds to treatment of the GPG with 6 N hydrochloric acid at room temperature for half an hour. This rate appears to be somewhat higher than that reported by SCHMIDT *et al.*[19] for GPE and should be anticipated for a bis-α-hydroxy ester of phosphoric acid.

The phytosynthesis of the phosphatidyl glycerols probably follows the pathway established for lecithin and phosphatidyl ethanolamine by KENNEDY AND WEISS[4]. The series of reactions would be represented by the following equations:

$$\text{Dihydroxyacetone phosphate} \xrightarrow[\text{DPNH}]{\text{"}\alpha\text{-Glycerophosphate dehydrogenase"}} \text{D-Glycero-1-phosphate}$$

$$\text{D-Glycerol-1-phosphate} + \text{CTP} \xrightarrow{\text{"Phosphoglycerol cytidylyl transferase"}} \text{CDPGlycerol} + \text{POP}$$

$$\text{D-2,3-Diglyceride} + \text{CDPGlycerol} \longrightarrow \text{Phosphatidyl glycerol} + \text{CMP}$$

The symmetry of diglycerophosphate suggests that some reservations may be necessary in considering this mechanism and that it may play a more important role in lipid biosynthesis than is immediately apparent. The concentration of free GPG in *Scenedesmus*[2] can be extremely high ($10^{-3}M$) but decreases rapidly during photo-

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synthesis. Experiments to evaluate its potentialities as a substrate for acylation are in progress. The possible importance of α-bisphosphatidic acids as diglyceride donors is obvious. The acylated GPG lipids may well provide the orientation essential in synthesis of both the triglycerides and the glycerolphosphatides.

The symmetry, simplicity and acid lability of the phosphatidyl glycerols appears to have delayed their recognition by classical methods. These very properties of the phosphatidyl glycerols may well be the key to their central importance in lipid metabolism.

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SUMMARY

1. The phospholipids of *Scenedesmus* and higher plants have been separated into at least three fractions by two-dimensional paper chromatography. These have been deacylated to yield glycerophosphorylcholine, glycerophosphorylglycerol and glycerophosphorylinositol.

2. The phosphatidyl glycerol fraction has been identified by hydrolysis of the purified lipid to give glycerophosphorylglycerol in 95% yield.

3. The presence of two free hydroxyl groups in the phospholipid was demonstrated by its conversion to an isopropylidene derivative and by oxidation by lead tetraacetate.

4. These lipids possess almost half of the lipid phosphorus in some plants and appear to be ubiquitous in nature.

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