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ISOLATION AND CHEMICAL CHARACTERIZATION OF PHOSPHATIDYL GLYCEROL FROM SPINACH LEAVES

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SUMMARY

Pure phosphatidyl glycerol was obtained from spinach leaves after repeated chromatography on silica columns. Ascertainment of the configuration of the hydrolysis products formed by the action of phospholipases C (EC 3.1.4.3) and D (EC 3.1.4.4) demonstrated that this phospholipid is identical with 1,2-diacyl-glycerol-3-phosphoryl-1'-glycerol. Fatty acid analysis of several lipid fractions showed that the Δ^3-*trans*-hexadecenoic acid, present in the leaves, is concentrated almost exclusively in phosphatidyl glycerol. Degradation experiments with phospholipase A (EC 3.1.1.4) showed that this acid is located preferentially at the 2-ester position. A subfractionation of phosphatidyl glycerol was accomplished by thin-layer chromatography on silica plates impregnated with silver nitrate. A breakdown of the two fractions obtained with phospholipase A allowed the recognition of several molecular species, and 1-linolenoyl,2-Δ^3-*trans*-hexadecenoyl-glycerol-3-phosphoryl-1'-glycerol appeared to be the major species. The results were confirmed by hydrolysis of phosphatidyl glycerol with phospholipase C and separation on impregnated adsorbents of the diglycerides formed.

INTRODUCTION

Phosphatidyl glycerol was discovered by BENSON AND MARUO[1] and established to be a quantitatively important[2,3] and highly dynamic[4] phospholipid of chloroplasts. This type of phospholipid is abundant also in various species of bacteria[5-7], but only relatively low concentrations have been detected in animal tissues[8-10] *e.g.* rat-liver mitochondria. Although small quantities of pure phosphatidyl glycerol have been obtained from several sources[7,9], a detailed characterization of this lipid required the isolation of substantial amounts from green leaves[11]. In addition to the elucidation of the stereochemical configuration[12] and the fatty acid composition[13,14] a nearly complete characterization of the individual molecular species of phosphatidyl glycerol from spinach leaves has now been achieved.

EXPERIMENTAL

Preparation of lipid extract

Six kg of fresh leaves of spinach (*Spinacia oleracea*, var. glabra Mnch.) were washed, kept for 1 min in water of 95° in order to inactivate phospholipases, and homogenized with methanol in a Waring Blendor. The mixture (volume 5 l) was filtered through filter cloth and washed with methanol and methanol–chloroform (1:1, v/v) until the insoluble material remained colourless. The filtrate was extracted according to the method of BLIGH AND DYER[15], taking into account the volumes of the added solvents and the water content of the leaves. The chloroform layer (10 l) was brought to dryness at a temperature below 37° in a rotary evaporator. The residue, weighing about 40 g and containing 300 mg of phosphorus, was dissolved in 50 ml of ether and poured into 1 l of dry acetone saturated with magnesium chloride. Storage of this mixture for 16 h at −15° gave a precipitation of 94% of the total lipid phosphorus, together with about 30% of the galactolipids, mainly digalactosylglyceride, whereas most of the monogalactosylglycerides, pigments, neutral lipids and the remaining digalactosyl derivatives were recovered in the supernatant (compare also Fig. 1). The precipitate (weight 17 g containing 286 mg of lipid phosphorus) was centrifuged, dissolved in chloroform and stored for use. All manipulations were carried out in a nitrogen atmosphere.

Chromatography on silica columns

Lipids were separated on silicic acid (Mallinckrodt, analytical grade) sieved into the desired mesh number. The adsorbent was washed with chloroform and methanol, and activated by heating at 110° for 1 h. Columns were made from a thin slurry of silica in chloroform, and washed with chloroform. The sample was dissolved in chloroform, and introduced on to the column. Elution was carried out with chloroform and mixtures of chloroform and methanol. Fractions of 20 ml were collected under nitrogen, and their composition was checked by thin-layer chromatography using the microslide procedure according to PEIFER[16].

Thin-layer chromatography

Preparative thin-layer chromatography was carried out on plates (20×5 cm), covered with a layer of 1 mm silica gel G-Stahl (Merck, Darmstadt). Silica plates impregnated with silver nitrate were prepared as described by BARRET *et al.*[17]. The separation of phospholipids on silver nitrate-impregnated plates was improved by adding Celite, using a suspension of 17 g Celite, 3 g of calcium sulphate and 40 g of silica gel G in 100 ml of a 19% solution of silver nitrate. All adsorbents were previously washed with chloroform–methanol (1:1, v/v), and the chromatoplates were activated by heating at 110° for 1 h, immediately before use. About 5–10 mg of the sample was applied to the plates. Spots were visualized under ultraviolet light, after spraying with a solution of 0.01% rhodamine 6 G in water. The spots were scraped from the glass plate, and lipids were extracted with methanol and methanol–chloroform (1:1, v/v), and subsequently treated according to the procedure of BLIGH AND DYER[15]. All manipulations were carried out in a nitrogen atmosphere. The individual lipids were identified by staining replicate chromatoplates with a ninhydrin reagent, the periodate–Schiff reagent[18], and by treatment with 50% sulfuric acid, which

caused typical reactions during the first minutes of charring[3], making it possible to identify the different classes of lipids. A solution of 10% of phosphomolybdic acid in ethanol was used to detect compounds on silver nitrate-impregnated plates.

Two-dimensional silica thin-layer chromatography was applied, using as solvent systems consequently chloroform–methanol (7:2, v/v) and diisobutylketone–acetic acid–water (40:25:3.7, v/v). (See ref. 3). The two-dimensional chromatographic separation of plant lipids was recently also published by LEPAGE[19].

Phospholipid hydrolysis

Mild alkaline hydrolysis of phospholipids was carried out according to the method of DAWSON[20] as modified by BENSON[21]. Extraction of the fatty acids was performed with isobutanol and chloroform. The water-soluble hydrolysis products were visualized on two-dimensional paper chromatograms by spraying with the periodate–Schiff reagent[18].

Chemical analyses

Phosphorus assays[22] and determinations of esterified [23] and unesterified[24] glycerol were carried out by established procedures. Quantitative periodate oxidation of phosphatidyl glycerol was performed according to KENNEDY *et al.*[10]. The determination of glycerol-3-phosphate (L-α-glycerophosphate) was essentially that described by BUBLITZ AND KENNEDY[25].

Gas-liquid chromatography

Fatty acid methyl esters were separated by gas-liquid chromatography using an F & M instrument equipped with flame ionization detector with a temperature of 197° and a 2-m column of 10% polyethylene glycol adipate on celite. The amounts of fatty acids were expressed as percentages of the total after measurement of the peak areas.

Enzymic hydrolysis of phosphatidyl glycerol

The incubations were carried out in a nitrogen atmosphere under shaking at room temperature. Saturated phosphatidyl glycerol, having a poor solubility in organic solvents, was suspended in ether by ultrasonic vibration.

Hydrolysis by phospholipase A (EC 3.1.1.4). An amount of 5 mg of substrate was dispersed or dissolved in 5 ml of ether and shaken with 0.5 ml of 0.1 M borate buffer (pH 7.0) containing calcium acetate and 1 mg of *Crotalus adamanteus* venom (Light and Co., Colnbrook). Within 2 h hydrolysis was found to be complete. The incubation mixture was brought to dryness under reduced pressure, and the residue was taken up in 1 ml of chloroform–methanol (1:1, v/v).

Hydrolysis by phospholipase C (EC 3.1.4.3). Incubation of 5 mg substrate in 1 ml of ether was carried out with 0.3 ml of a solution of a crude enzyme preparation. The phosphatidyl glycerol was usually completely hydrolysed within 1 h. The water-soluble and lipid-soluble products were separated by the method of BLIGH AND DYER[15]. The phospholipase C preparation was obtained from a aerobic culture of *Bacillus cereus*[26] grown for 18 h at 37° at pH 7. The culture was centrifuged 20 min at 18000 × *g*. To 100 ml of the clear supernatant 60 g of ammonium sulfate was added. The precipitate obtained after standing for 16 h at 0° was centrifuged at 0° and dis-

solved in 2 ml of 0.1 M Tris buffer (pH 7.2). The solution was made clear by centrifugation. It could be stored for at least 3 months at 0° without any significant loss of activity.

Hydrolysis by phospholipase D (EC 3.1.4.4). To 5 mg of substrate dispersed in 5 ml of ether was added 1 ml of 0.1 M acetate buffer (pH 5.6), 1 ml of 0.02 M calcium chloride and 4 mg of a crude phospholipase D preparation (C. F. Boehringer und Söhne, Mannheim) dissolved in 0.2 ml of water. Complete hydrolysis was obtained after a 4-h incubation period. The water-soluble and lipid-soluble products were separated in the usual way[15].

The quantitative assays were corrected for non-enzymic degradation.

TABLE I

CHROMATOGRAPHY ON A SILICIC ACID COLUMN OF ACETONE-INSOLUBLE LIPIDS FROM SPINACH LEAVES

*Combined tubes**	*Fraction*	*Chloroform–methanol (v/v)*	*Thin-layer chromatographic analysis***					
			Phosphatidic acid	*Monogalactosyl glyceride*	*Phosphatidyl glycerol*	*Digalactosyl glyceride*	*Phosphatidyl ethanolamine*	*Phosphatidyl choline*
1–100	1	100:0						
101–200	2	98:2	Trace					
201–250	3	96:4		++				
251–300	4	96:4		+	+			
301–325	5	92:8			+++			
326–350	6	92:8			++	+	Trace	
351–450	7	88:12			Trace	+++	+	
451–550	8	80:20				+	+++	
551–650	9	50:50					+	++
651–750	10	0:100						++

* Each tube contained 20 ml.

** +++, highly positive; ++, positive; +, slightly positive.

RESULTS AND DISCUSSION

The isolation of phosphatidyl glycerol

To obtain a good separation of the lipids present in the acetone precipitate (compare Fig. 1 B) use was made of chromatography on silica columns. This crude mixture, containing 286 mg lipid phosphorus was introduced on to a column containing 480 g of silica (60–100 mesh, column 56 × 5 cm). The results of the separation are indicated in Table I. In nearly all fractions some minor unidentified components were present. Elution with methanol–chloroform (2:98, v/v) yielded phosphatidic acid, corresponding to 0.8% of the total amount of phosphorus applied to the column. This observation supports the results of other recent investigations[2,3,4,27] and indicates that only small quantities of this phospholipid exist in green leaves. Fractions 3, 8 and 9 eluted with a higher concentration of methanol were subjected to preparative thin-layer chromatography to isolate monogalactosylglyceride, phosphatidyl ethanolamine and phosphatidyl choline respectively, using as development agent chloroform–methanol–acetic acid (65:25:4, v/v/v). Fatty acid analyses on these lipids are described elsewhere in this paper. Fractions 4, 5 and 6 were combined and denoted "PG concentrate". As demonstrated on thin-layer chromatograms (Fig. 1 C)

these fractions contained phosphatidyl glycerol, some galactolipids, a trace of phosphatidyl ethanolamine and three or four other minor components, not identified in the present study. This phosphatidyl glycerol concentrate (weight 2.1 g, containing 55 mg lipid phosphorus) contained 1.2 g of phosphatidyl glycerol, being nearly all of the phosphatidyl glycerol originally extracted. These results suggest that about 20% of the phospholipids from spinach leaves consisted of phosphatidyl glycerol. This observation is in agreement with the results of other investigators[2,4,27], who found about the same content of phosphatidyl glycerol in various green leaves.

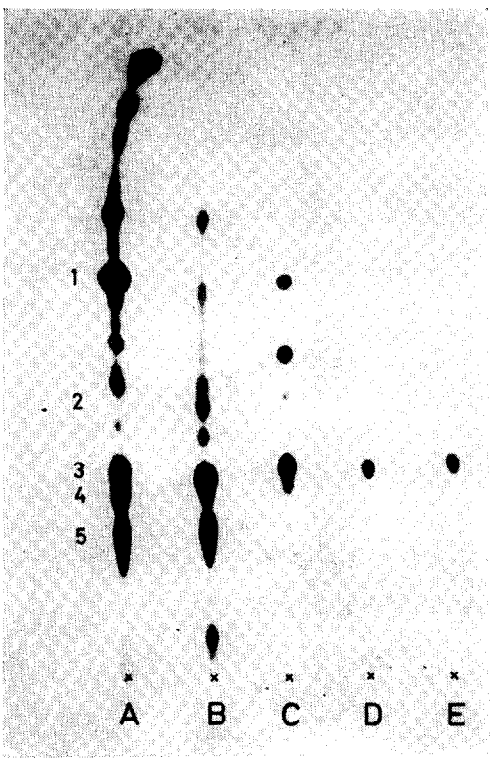

Fig. 1. Thin-layer chromatogram of some lipid fractions representing different stages in the isolation of phosphatidyl glycerol. A, total lipid extract; B, acetone-insoluble material; C, phosphatidyl glycerol concentrate, obtained after one column separation; D, pure isolated phosphatidyl glycerol; E, synthetically-prepared phosphatidyl glycerol. Some of the major lipids present are monogalactosylglyceride (1), phosphatidyl ethanolamine (2), phosphatidyl glycerol (3), digalactosylglyceride (4) and lecithin (5). Solvent system: chloroform–methanol–concentrated ammonia (70:20:2, v/v/v).

To obtain pure phosphatidyl glycerol suitable aliquots of the phosphatidyl glycerol concentrate were subjected to rechromatography on a column containing 30 g of silica (140–200 mesh, column 30 × 1,7 cm) which was loaded with a lipid sample containing 4.4 mg of lipid phosphorus. After elution with chloroform and methanol–chloroform (2:98, v/v), phosphatidyl glycerol was recovered with methanol–chloroform (4:96, v/v). The purity of the several fractions obtained was verified in the first instance by thin-layer chromatography, using a synthetic substance as reference (Figs. 1D and 1E). The fractions were combined, and the total lipid phosphorus

content of such preparations amounted to 2.1 mg, corresponding to a yield of pure phosphatidyl glycerol of 105 mg/kg of wet leaves. Assuming that the phosphatidyl glycerol concentrate contained all the phosphatidyl glycerol present in the leaves, it appears that about 50% of the total phosphatidyl glycerol available was obtained in a pure state. The fatty acid composition of different pure phosphatidyl glycerol fractions eluted from the column were always identical to each other, demonstrating that the isolated product was a suitable representative of the total phosphatidyl glycerol extracted from the leaves.

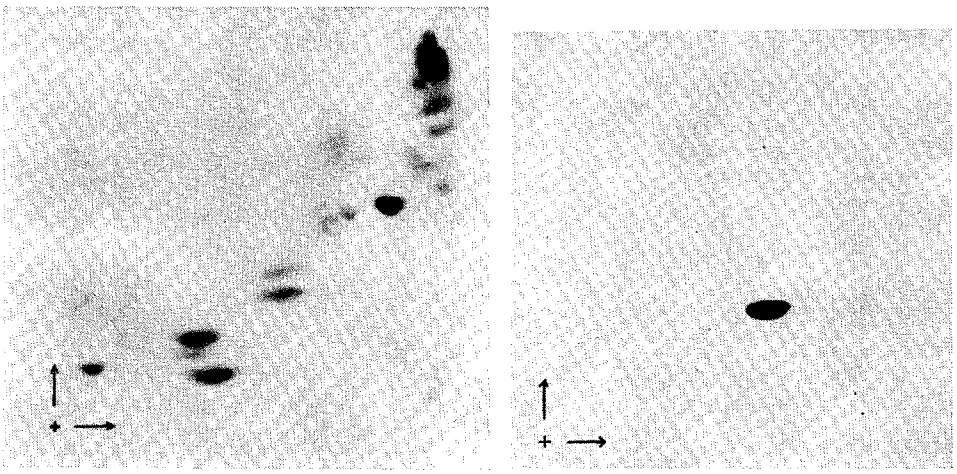

Fig. 2. Two-dimensional thin-layer chromatograms of total lipid extract (left) and pure phosphatidyl glycerol (right) from spinach leaves. Solvent system: horizontal direction, chloroform–methanol (7:2, v/v); vertical direction, di-isobutylketone–acetic acid–water (40:25:3.7, v/v).

When the latter separation was carried out on a seven times greater scale, the results were less reproducible, but this technique was successful when the lipid mixture was first hydrogenated. Catalytic hydrogenation of the phosphatidyl glycerol concentrate was effected in methanol with platinic oxide. The hydrogenated lipids, dissolved in chloroform, and containing 30 mg lipid phosphorus, were applied to a column of 320 g of silica (140–200 mesh, column 135 × 3.0 cm). Elution was performed as described above. About 350 mg pure phosphatidyl glycerol was recovered from the methanol–chloroform (4:96, v/v) eluant, the yield per kg leaves being the same as that of the smaller scale isolation of unhydrogenated phosphatidyl glycerol. However, further improvement of the yield was possible by low temperature crystallization of some impure fractions from methanol–chloroform (9:1, v/v), which supplied an additional 50–100 mg of pure hydrogenated phospholipids.

The purity of the isolated phosphatidyl glycerol has been tested by different analytical methods. Two-dimensional thin-layer chromatograms (Fig. 2) of phosphatidyl glycerol revealed, after spraying with 50% sulfuric acid and successive charring, only one spot, again corresponding exactly with a synthetic specimen. The separation of various types of plant lipids on these plates was highly effective, as may be demonstrated by a chromatogram of a total lipid extract (Fig. 2) giving about 25 spots. Mild alkaline hydrolysis of phosphatidyl glycerol gave a water-soluble product,

which showed, when applied to a two-dimensional paper chromatogram, only one intensive spot (Fig. 3) having the R_F values of diglycerophosphate[4]. Chemical analyses of the isolated products (Table II) confirmed their purity. The phosphorus content of the hydrogenated products was very satisfactory. The non-hydrogenated phospholipid gave somewhat lower values, probably due to residual water, which is difficult to remove, or perhaps to some oxidation of the dried product. The glycerol/phosphorus ratio, which was found by determining both the glycerol and phosphorus content of the lipid hydrolysate[23], after its extraction with chloroform, was 2:1. The number of adjacent hydroxyl groups per molecule of phosphatidyl glycerol was found to be equal to unity. In the case of non-hydrogenated phosphatidyl glycerol this determination was disturbed by the presence of polyenoic fatty acid constituents.

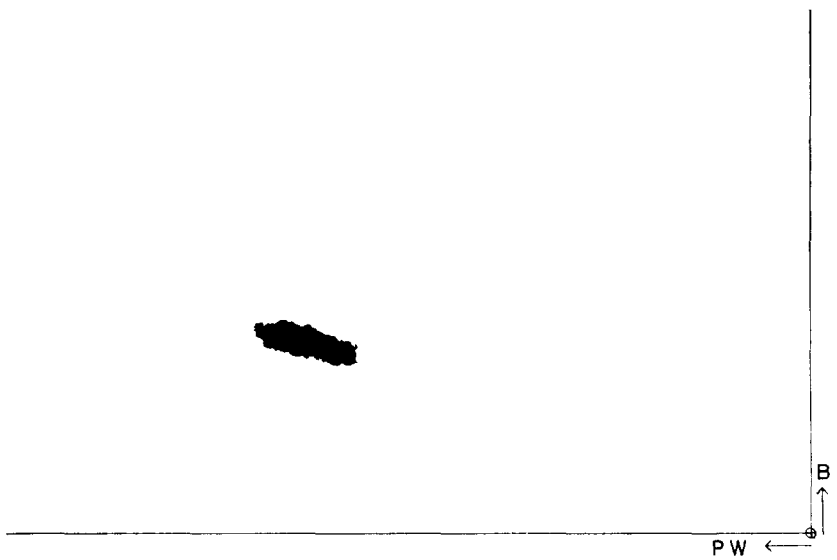

Fig. 3. Two-dimensional paper chromatogram of the water-soluble hydrolysis product of isolated phosphatidyl glycerol *viz.* diglycerophosphate. Solvent system: phenol–water (PW) and *n*-butanol–propionic acid–water (71:36:50, v/v/v) (B).

TABLE II

ANALYTICAL DATA OF PHOSPHATIDYL GLYCEROL FROM SPINACH LEAVES

Analysis	*Unhydrogenated*	*Hydrogenated**	*Calculated*
P (%)**	3.70	3.89	4.10***
	3.72	3.92	
Mole ratio glycerol: P	1.92	1.96	2.00
	2.00	2.04	
Mole ratio vicinal OH-groups: P		1.02	1.00

* Isolated from a hydrogenated mixture of lipids as described in the text.
** Determined on samples dried over phosphorus pentoxide *in vacuo*.
*** This value was calculated by taking a molecular weight of 750, which was derived after analysis of the fatty acids.

The configuration of phosphatidyl glycerol

Both phosphatidyl glycerol in its native state and the hydrogenated product were found to be completely hydrolysed by phospholipase A, C and D. The reactions are indicated in Fig. 4.

The enzymic breakdown by phospholipase A was demonstrated by means of thin-layer chromatography (Fig. 5). According to expectation, the lysocompound formed had a lower R_F value than the intact phospholipid, and gave a positive reaction with the periodate–Schiff reagent[18], while the second spot appeared to consist of free fatty acids.

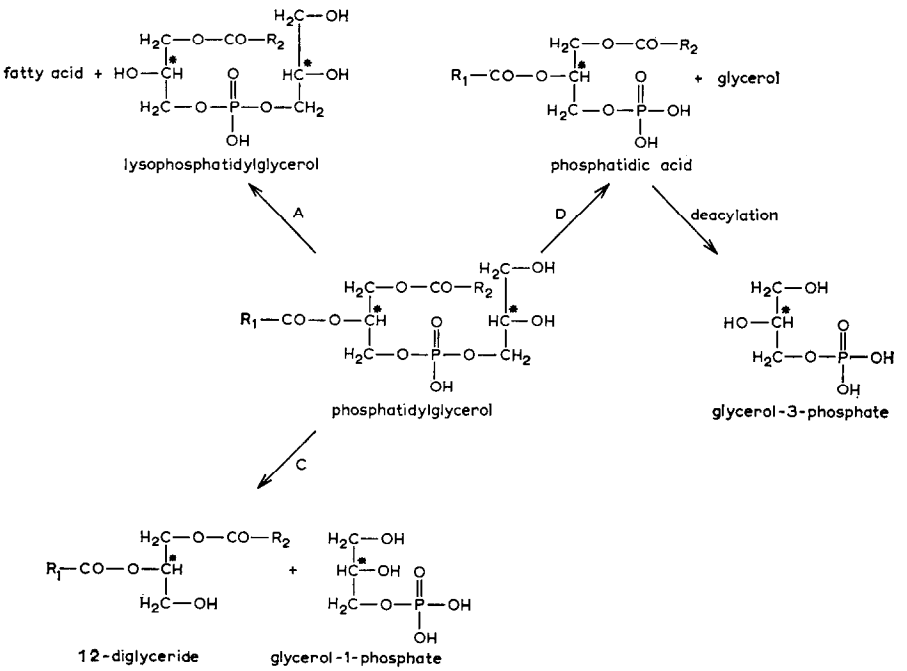

Fig. 4. Scheme of the hydrolysis of phosphatidyl glycerol by phospholipase A, C and D. R stands for the apolar moiety of fatty acids. Optically active carbon atoms are indicated by an asterisk.

Phospholipase C furnished a water-soluble product, which was paper-chromatographically identical to 1- or 3-glycerolphosphate, when *tert.*-butanol–picric acid–water[28] (80:4:20, v/v/v) was used as a solvent system. Quantitative determinations of phosphorus, vicinal hydroxyl groups[24] and glycerol 3-phosphate (L-α-glycerophosphate) are enumerated in Table III. Aliquots of standard solution of glycerol 3-phosphate added to the incubation mixture were quantitatively recovered. The results demonstrated that the water-soluble hydrolysis product was undoubtedly identical to glycerol 1-phosphate (D-α-glycerophosphate). The lipid-soluble layer was shown by thin-layer chromatograms to contain a diglyceride carrying two adjacent fatty acid ester linkages. The chromatographic comparison with 1,2- and 1,3-digly-

cerides is reproduced in Fig. 6. Incubation of 100 mg of hydrogenated* phosphatidyl glycerol with phospholipase C yielded 60 mg of pure diglyceride, having an optical activity $[\alpha]_D^{20}$ – 3.3 in chloroform (*c*, 2.0). The optical rotation of this diglyceride, containing over 95% palmitic acid and stearic acid, was close to the values reported

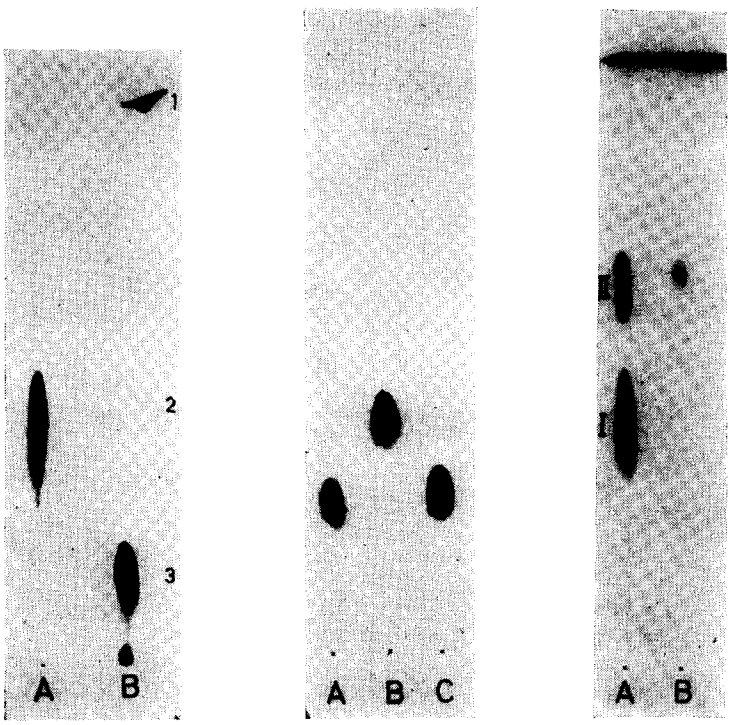

Fig 5. Demonstration of the hydrolysis of phosphatidyl glycerol by phospholipase A by means of thin-layer chromatography; before (A) and after (B) incubation. Solvent system: chloroform–methanol–acetic acid (70:20:2, v/v/v). The spots represent fatty acids (1), phosphatidyl glycerol (2) and lysophosphatidyl glycerol (3).

Fig. 6. Identification of the diglyceride (A), obtained by the action of phospholipase C on isolated phosphatidyl glycerol. Reference substances are 1-palmitoyl, 3-oleoyl glycerol (B) and 1,2-distearoyl-glycerol (C). Thin-layer chromatograms. Solvent system: ether–hexane (3:7, v/v).

Fig. 7. Subfractionation of pure phosphatidyl glycerol (A) from spinach leaves into Fractions I and II, by means of thin-layer chromatography on silica, impregnated with silver nitrate. Phosphatidyl glycerol with saturated fatty acids as a control (B). Solvent system: chloroform-ethanol-water (65:30:3.5, v/v/v).

by BAER AND KATES[29] for synthetic 1,2-dipalmitine and 1,2-distearine. Hence, it can be concluded, that the diglyceride obtained as a hydrolysis product is a 1,2-diglyceride (D-α, β diglyceride).

The action of phospholipase D yielded a water-soluble product, which was

* For this purpose use was made of a hydrogenated substrate since the optical activity of diglyceride from phosphatidyl glycerol in the native state could easily be influenced by some oxidation of the unsaturated fatty acids.

TABLE III

ANALYSIS OF THE WATER-SOLUBLE FRACTION OBTAINED AFTER HYDROLYSIS OF PHOSPHATIDYL GLYCEROL BY PHOSPHOLIPASE C

Analysis	*Before hydrolysis substrate*	*After hydrolysis water-soluble fraction*
P (μatoms)	2.25	2.20
adjacent OH-groups (μmoles)		1.90
glycerol 3-phosphate (μmoles)		0.00

TABLE IV

ANALYSIS OF THE PRODUCTS OBTAINED FROM PHOSPHATIDYL GLYCEROL BY THE ACTION OF PHOSPHOLIPASE D

Analysis	*Before hydrolysis substrate*	*After hydrolysis*	
		Water-soluble fraction	*Lipid-soluble fraction**
P (μatoms)	6.0	0.0	5.1
free glycerol (μmoles)	—	6.3	—
glycerol 3-phosphate (μmoles)	—	0.0	4.8

* After alkaline hydrolysis and subsequent removal of lipids.

shown by means of paper chromatography to be identical with glycerol. Actually a quantitative assay showed that an equimolecular quantity of glycerol was liberated by this enzyme (Table IV). The lipid-soluble fraction contained phosphatidic acid, as shown on thin-layer chromatoplates. This fraction was subjected to alkaline hydrolysis by treatment with 2 ml of 1 N KOH in methanol for 30 min at 55°. The hydrolyzate was cooled, 3 ml of water was added, and the pH adjusted to about 8.5. After extraction of the lipids and removal of the methanol, the water-soluble products were analysed for phosphorus and glycerol 3-phosphate (L-α-glycerophosphate). The yield of phosphorus was 85% calculated on the quantity of incubated phosphatidyl glycerol, while the ratio of atoms phosphorus to moles glycerol 3-phosphate was close to unity (Table IV). Alkaline hydrolysis of phosphatidic acid has been shown not to cause migration of the phosphate moiety or racemization[30-32], our results being in good agreement with these observations.

The phosphatidyl glycerol molecule, containing two asymmetric centres, can exist theoretically in any of four stereoisomeric forms. BAER AND BUCHNEA[33] synthesized 1,2-diacyl-glycerol-3-phosphoryl-3'-glycerol (L-α-phosphatidyl-L-glycerol), while BENSON AND MIYANO[34] considered it more likely that the compound present in photosynthetic tissues is identical with 1,2-diacyl-glycerol-3-phosphoryl-1'-glycerol (L-α-phosphatidyl-D-glycerol). The experiments described above unambiguously establish the latter configuration to apply for phosphatidyl glycerol from spinach leaves (Fig. 4). As regards the configuration of the phosphatidyl moiety the experiments with phospholipase A from snake venom corraborate the results obtained with phospholipase C and D, since the former enzyme acts on diacyl derivatives of glycerol 3-phosphate but not on those of glycerol 1-phosphate[35]. That both glycerol moieties have an opposite configuration, as found after degradation experiments involving phospholipase C and D, was further supported by comparison of the optical rotations of isolated and synthetic substances.

Furthermore the results are in accordance with biosynthetic investigations of KENNEDY *et al*[10] indicating a similar configuration for phosphatidyl glycerol from rat liver.

The fatty acid composition of phosphatidyl glycerol and some other leaf lipids

The chloroform extract of spinach leaves obtained before the precipitation in acetone showed a fatty acid composition (Table V) in good agreement with the fatty acid pattern of the total lipid fraction found by others [14,36,37]. A high content of linolenic acid is obvious. A small percentage of a hexadecenoic acid was present which, in good agreement with the studies of DEBUCH[37] and KLENK AND KNIPPRATH[38], turned out to be a Δ^3-*trans*-hexadecenoic acid. Confirmation of this structure was obtained after the isolation of its methyl ester by means of gas-liquid chromatography. Oxidative degradation[39] yielded tridecanoic acid, catalytic hydrogenation produced palmitic acid, and an infrared spectrum clearly showed the presence of a *trans* bond at a frequency of 965 cm^{-1}. Also a hexadecatrienoic acid was found. The data available so far[40–43] suggest that this type of fatty acid occurs widely in plants.

TABLE V

THE FATTY ACID COMPOSITION OF PHOSPHATIDYL GLYCEROL AND SOME OTHER LIPIDS FROM SPINACH

Acids	*Total lipid extract*	*Phosphatidyl choline*	*Phosphatidyl ethanolamine*	*Monogalactosyl-glyceride*	*Phosphatidyl glycerol*		
					Total	*1-Position*	*2-Position*
16:0*	17.0	21.2	26.5	9.7	20.0	32.4	11.1
16:1 *trans*	2.4	—	—	—	31.7	0.5	58.9
16:3	7.4	—	—	25.3	—	—	—
18:0	0.5	1.3	2.2	—	0.6	1.2	—
18:1	6.0	15.8	6.9	—	2.6	2.4	3.1
18:2	14.6	31.6	42.1	—	8.0	2.1	14.5
18:3	51.4	30.1	22.4	65.1	37.1	61.4	12.4

* The number before the colon denotes chain length; that after it the number of double bonds.

It was thought to be of interest to study the distribution of the various fatty acids among different lipid classes. Lecithin, phosphatidylethanolamine and monogalactosylglyceride were purified by thin-layer chromatography as described earlier in this paper. Lecithin was found to possess a fatty acid composition very similar to that reported by SASTRY AND KATES[44] for lecithin from runnerbean leaves. Phosphatidyl ethanolamine is noted for its rather high content of linoleic acid. However, neither phospholipid contained detectable amounts of the C_{16} *trans*-monoenoic or the C_{16} trienoic acid. The monogalactosylglyceride was found to contain about 25% hexadecatrienoic acid, and in spinach leaves this acid appears to be concentrated mainly in this lipid species. A similar observation was recently made by ALLEN *et al.*[14]. Phosphatidyl glycerol possessed also an unique fatty acid composition[13], as much as 30% of the fatty acid constituents consisting of Δ^3-*trans*-hexadecenoic acid, whereas no measurable quantities of this fatty acid could be detected in any of the other lipid types. Recently other investigators[14,45] have made similar observations. The positional distribution of the fatty acids was investigated after complete breakdown of phosphatidyl glycerol by the action of phospholipase A from snake venom,

which liberates specifically the fatty acids from the 2-position. After separation of the two hydrolysis products on thin-layer chromatograms (Fig. 5) analyses showed that the Δ^3-*trans* hexadecenoic acid was located exclusively at the 2-positon (Table V) while a predominant part of the linolenic acid appeared to occupy the 1-position. This distribution differs significantly from that usually found in other phospholipids *e.g.* lecithin from runner-bean leaves was reported to contain the poly-unsaturated fatty acids mainly at the 2-position[44].

The data available so far indicated that phosphatidyl glycerol and monogalactosylglyceride are intimately linked with Δ^3-*trans*-hexadecenoic acid and hexatrienoic acid respectively. Taking into account the observations of several research groups[2,3,4,46] it appears that these particular lipid combinations are specifically located in the chloroplast. In this context it is of interest that current investigations showed that in etiolated cells of *Euglena gracilis* having a very low content of phosphatidyl glycerol the Δ^3-*trans*-hexadecenoic acid was lacking, but this lipid combination was present in green cells.

Subfractionation of phosphatidyl glycerol

During the past few years[17,47–49] silver nitrate-impregnated silica has been extensively used to separate lipids according to the degree of saturation of their fatty acid constituents. As regards the fractionation of phospholipids reference can be made only to a study on lecithins[50].

This principle has been applied now to subfractionate pure phosphatidyl glycerol on chromatoplates. Two fractions were obtained (Fig. 7), denoted as PG I and

TABLE VI

MOLECULAR SPECIES OF PHOSPHATIDYL GLYCEROL FROM SPINACH LEAVES

Fatty acid analyses are given of two fractions of phosphatidyl glycerol (PG I and PG II) separated on impregnated adsorbents. The results of the breakdown of these fractions by phospholipase A, and experiments involving phospholipase C and separation of diglycerides (Table VII) have been used to derive the occurrence of several fatty acid combinations.

Fatty acid	*PG I*			*PG II*	
16:0	16.3			42.9	
16:1 *trans*	40.6			15.5	
18:0	—			2.9	
18:1	—			9.4	
18:2	—			29.4	
18:3	43.1			—	
			phospholipase A treatment		
	1-position	*2-position*		*1-position*	*2-position*
16:0	22.1	11.2		73.3	15.2
16:1 *trans*	—	73.2		—	26.6
18:0	—	—		6.8	2.0
18:1	—	—		8.9	10.8
18:2	—	—		11.0	45.4
18:3	77.9	15.6		—	—
	P-G* 16:1 *trans* 18:3 *α*	P-G 18:3 16:0 *β*	P-G 16:0 18:3 *γ*	P-G 18:2 16:0 *δ*	P-G 16:1 *trans* 16:0 *ε*

* This shorthand formula stands for 1-linolenoyl, 2-Δ^3-*trans*-hexadecenoyl-phosphatidyl glycerol.

PG II, the former fraction having the lowest mobility. By virtue of the lipid-phosphorus content, their ratio was found to be PG I : PG II = 77 : 23. Fatty acid analyses of both subfractions are listed in Table VI. According to expectation, fraction PG I contained all the linolenic acid present in phosphatidyl glycerol, while the other C_{18}-acids including linoleic and oleic acid were exclusively found in fraction PG II. Palmitic acid and Δ^3-*trans*-hexadecenoic acid were present in both spots. In order to find out the positional distribution of the fatty acids, both fractions were subjected to hydrolysis by phospholipase A.

The results, demonstrated in Table VI, allowed a first approximation of the occurrence of different fatty acid combinations in phosphatidyl glycerol, neglecting the minor fatty acids stearic and oleic acid. Fraction PG I, appeared to contain linolenic acid predominantly at the 1-position and Δ^3-*trans*-hexadecenoic acid exclusively at the 2-position. The high content of both fatty acids in this fraction clearly indicates that this combination (denoted as α, Table VI) represents the prevailing molecular species of phosphatidyl glycerol. Apart from this compound containing four double bonds in the acyl chains, theoretically the existence of at least 5 other combinations has to be envisaged. However, dilinolenoyl- and dipalmitoyl-phosphatidyl glycerol, having 6 and 0 double bonds per molecule respectively, are not likely to be present in this fraction, because such compounds are expected to have a different mobility on the impregnated absorbents used. For that reason it is more feasible that in this fraction palmitic acid is combined with linolenic acid as well giving the molecular species β and γ (Table VI). However, the total linolenic acid content of this fraction is not sufficient to account for the three combinations given and, as will be discussed below, another species *viz.* 1-palmitoyl,2-Δ^3-*trans*-hexadecenoyl phosphatidyl glycerol may be present to a small extent in this fraction as well. Taking into consideration the ratio of the fractions PG I and PG II, it can be concluded that 1-linolenoyl, 2-Δ^3-*trans*-hexadecenoyl phosphatidyl glycerol (α) is quantitatively the most important component, constituting nearly 50% of the phosphatidyl glycerol species from spinach leaves.

TABLE VII

FATTY ACIDS OF DIGLYCERIDES OBTAINED FROM PHOSPHATIDYL GLYCEROL AFTER HYDROLYSIS BY PHOSPHOLIPASE C

The hydrolysis products were fractionated by thin-layer chromatography on silicic acid impregnated with silver nitrate.

Fatty acids	*Fraction**				
	a	*b*	*c*	*d*	*e*
16:0	17.6	49.8	42.7	49.8	60.0
16:1 *trans*	42.6	10.0	15.8	25.3	40.0
18:0	—	—	13.8	8.5	—
18:1	—	—	27.7	16.3	—
18:2	—	41.0	—	—	—
18:3	39.8	—	—	—	—

* Fractions have R_F values, from left to right, in increasing order.

Fraction PG II revealed a relatively high content of palmitic acid at the 1-position, and of linoleic acid at the 2-position, suggesting the occurrence of δ in phosphatidyl glycerol though both fatty acids may be present in other combinations

as well. The distribution of various fatty acid constituents among both ester positions in fractions PG II forces us to take into consideration the occurrence of numerous molecular species, but the data obtained by the method used did not allow final conclusions. The assumption that 1-palmitoyl,2-Δ^3-*trans*-hexadecenoyl phosphatidyl glycerol (Σ)represents one of the molecular species was supported by analysing the area located on the chromatoplate between the two major spots I and II. This very minor fraction appeared to contain palmitic acid and Δ^3-*trans*-hexadecenoic acid in a ratio exactly 1 : 1. The trailing of this compound (Σ) may be caused by a poor solubility in the solvent system used and its presence in both phosphatidyl glycerol fractions perhaps could explain some of the difficulties encountered when making the balance of PG I.

Although the results obtained by subfractionation of phosphatidyl glycerol on an adsorbent impregnated with silver nitrate, and successive breakdown by phospholipase A furnished some information about the major molecular species present, an additional approach was highly desirable. Another possibility for investigating the fatty acid distribution of phosphatidyl glycerol was given by utilizing its susceptibility to phospholipase C and to separate the diglycerides formed by thin-layer chromatography on silica impregnated with silver nitrate. Using as a developing solvent chloroform–ethanol (95:5, v/v) five fractions could be obtained showing the fatty acid composition listed in Table VII. Fraction a, having the lowest mobility, was the major spot and was found to have a fatty acid composition similar to that of the fraction PG I. This observation confirms the conclusion that 1-linolenoyl,2-Δ^3-*trans*-hexadecenoyl phosphatidyl glycerol is quantitatively a most important species. Fraction b contained, besides a small amount of Δ^3-*trans*-hexadecenoic acid, palmitic acid and linoleic acid as fatty acid constituents. In combination with the known positional distribution of these fatty acids in fraction PG II, the results obtained on these diglycerides confirm the existence of 1-palmitoyl, 2-linoleylphosphatidyl glycerol (δ). Fractions c and d did not allow a further unravelling of distinct molecular species, but a degradation of these and the other diglycerides with pancreatic lipase (EC 3.1.1.3) may be rewarding. Fraction e confirmed the occurrence of 1-palmitoyl,2-Δ^3-*trans*-hexadecenoyl phosphatidyl glycerol (ε), which species was already identified from the phospholipid analysis. As in the case of the corresponding phosphatidyl glycerol, this diglyceride revealed a trailing on the chromatograms, which perhaps accounted for the fact that both fatty acids were recovered in all diglyceride fractions.

In short it can be concluded that the occurrence of the molecular species α, δ and ε in phosphatidyl glycerol from spinach leaves was established by this approach. The existence of β and γ is likely but needs further confirmation. Taking into consideration the percentage of the various fatty acids, it can be calculated that these five components α, β, γ, δ and ε constitute at least 90% of the phosphatidyl glycerol preparation investigated. The techniques used could probably be further improved, and in principle are suitable for the recognition and determination of the individual molecular species present in other types of phosphoglycerides.

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