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Composition of cabbage leaf phospholipids*

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SUMMARY

An attempt has been made to resolve the phospholipids of cabbage leaf by chromatography on silicic acid. The components include phosphatidylglycerol and an unknown glycerolphospholipid. The phospholipids were of fairly uniform fatty acid composition, containing predominantly palmitic, linoleic, and linolenic acids.

Several metabolic studies indicate that there is a rapid turnover of the nitrogen-free phospholipids such as simple or complex phosphatidic acids and inositides (1, 2, 3). The fatty acid composition of the members of this group so far examined is unusual and suggests a high selectivity in their biosynthesis. Thus cardiolipin, the complex phosphatidic acid isolated by Pangborn (4) from ox heart, contains only unsaturated acids, mainly linoleic; there is evidence in favor of the structure *bis* (diacylglycerophosphoryl) glycerol for this compound (5, 6). The phosphomonoinositides isolated from wheat germ, ox heart, and ox liver (7, 8) contained equimolar amounts of a saturated acid (either palmitic or stearic) and unsaturated acids with high iodine numbers.

The fatty acid composition of the phosphatidic acid found in animal tissues (2, 9) is not known. Phosphatidic acid with a high proportion of unsaturated acids was isolated from cabbage leaf by Chibnall and Channon (10), but subsequent work indicates that this was largely an artifact due to the action of a phospholipase which can split the nitrogenous base from phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (11, 12, 13). The discovery of phosphatidylglycerol as a major component of plant leaf phospholipids (14), and subsequently in trace amounts in the rat (15), suggested a possible relationship to phosphatidic acid and cardiolipin. It therefore seemed of interest to re-examine the phospholipids of cabbage leaf and compare their fatty acid composition with those of animal tissues.

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METHODS

Phosphorus, amino-nitrogen, fatty acid ester, inositol, phosphomonoester, and choline-containing phospholipids were estimated as described by Gray and Macfarlane (16), and choline according to Wheeldon and Collins (17).

For glycerol in nitrogen- and inositol-free phospholipid, the sample was saponified by refluxing in 0.5 N NaOH in 50% (v/v) methanol for 4 hours, and after neutralization and extraction of fatty acids with diethyl ether, the glycerophosphate was hydrolyzed with bone phosphatase (16); glycerol was then determined by spectrophotometric estimation of periodate consumed in 20 minutes at room temperature, checked by colorimetric determination of formaldehyde with chromotropic acid. For glycerol in water-soluble esters of glycerophosphoric acid, the sample was first hydrolyzed to monoester in 6 N HCl at 100° for 2 hours. After removal of HCl by evaporation, the procedure was the same as for lipid samples.

Serine and ethanolamine were estimated as the dinitrophenyl derivatives as follows: Lipid samples (0.5 to 1.5 μmoles amino-N) were hydrolyzed in 2.0 ml 3 N HCl (10 N HCl diluted with dioxane) at 100° for 3 hours in a stoppered tube. After extraction three times with diethyl ether, the aqueous phase was taken to dryness at 80° to 90° under a stream of air, and water (0.30 ml), 0.1 N $NaHCO_3$ (0.20 ml), and 0.1 M 1-fluoro, 2,4-dinitrobenzene in dioxane (0.20 ml) were added. The reaction mixture was left at 70° to 80° for 1 hour, and after addition of two drops of 1 N HCl, was taken to dryness *in vacuo*. The residue was dissolved in 6 N HCl (0.20 ml) and extracted twice with petroleum ether (b.p. 40-60°; 3 to 5 ml) to remove

all 2,4-dinitrophenol and dinitrobenzene. The acid solution was taken to dryness at 80° to 90° under a stream of air (the acid refluxes initially, effecting thorough drainage of the tube), and the residue was transferred quantitatively in 0.05 to 0.10 ml methanol-ether 1/1(v/v) to Whatman No. 3 chromatography paper. Separation of the serine and ethanolamine derivatives was accomplished by ascending chromatography in a light-protected vessel, using the upper phase of the mixture water:pyridine:tert-amyl alcohol 5/1/5(v/v) (18). The appropriate areas were cut out and eluted by soaking 2 to 3 hours in 2.5 to 10 ml 0.1 N HCl in ethanol, in which solvent $\epsilon_{350\ m\mu}$ is 17,200 and 15,700 for the ethanolamine and serine derivatives, respectively. Blank values obtained by elution of corresponding areas after chromatography corresponded to 0.045 μmole ethanolamine and 0.028 μmole serine.

For the identification of phospholipids from glycerophosphoric esters formed on deacylation, mild alkaline hydrolysis of samples (1 mg P) was carried out by Dawson's (1) method. After neutralization with Amberlite® IRC-50, the hydrolyzate was extracted with ethyl ether, adding methanol to break emulsions, and taken to dryness at 40° *in vacuo*. The residue was dissolved in 1.0 ml water and samples taken for total phosphorus and phosphomonoester determinations. The remainder was reduced to 0.10 ml and samples of 3 to 6 μl chromatographed according to Dawson (1). Table 1 shows R_f values for the esters detected and for authentic markers. Phosphatidic acid was estimated as phosphomonoester formed on mild alkaline hydrolysis (see above).

TABLE 1. R_f VALUES OF GLYCEROPHOSPHATE DERIVATIVES

Ester	R_f(1) *	R_f(2) *
Glycerophosphoric acid (GP)	0.25	0.61
Polyglycerophosphoric acid (GPGPG) †	0.125	0.45
Glycerophosphorylglycerol (GPG) (spot C)	0.40	0.56
Unknown (spot D)	0.56	0.67
Glycerophosphorylinositol (GPI)	0.09	0.26
Glycerophosphorylserine (GPS)	0.20	0.41
Glycerophosphorylethanolamine (GPE)	0.67	0.41
Glycerophosphorylcholine (GPC)	0.90	0.41

* R_f values are for acid-washed Whatman No. 1 chromatography paper. (1) phenol-ammonia; (2) *tert*-butanol-trichloroacetic acid. Distance run by the solvent (ascending), 10 inches.

† Derived from cardiolipin.

Fatty acids of phospholipid samples (0.4 to 4.0 mg P) were saponified overnight at room temperature under nitrogen in 10 ml N NaOH. Under these conditions, phospholipid fractions from the silicic acid column dissolved to form clear solutions and the amount of fatty acids liberated agreed with the fatty acid ester value of the phospholipid, except in a few cases. After acidification with 1.5 ml 10 N HCl, the fatty acids were extracted with diethyl ether, washed, dried, and made to volume in chloroform. A sample of the chloroform solution was titrated potentiometrically in 10 ml pyridine with 0.01 N tetraethylammonium hydroxide dissolved in 2-ethoxy ethanol. The end point for 5 to 10 μmoles fatty acid was accurate to 5% to 2%. Bromine uptake was determined on a sample of the fatty acids solution by the method of Trappe (19). The remainder of the fatty acids was converted to methyl esters by refluxing in anhydrous methanolic HCl. Gas chromatographic analysis was carried out by Dr. G. M. Gray on Apiezon L and Reoplex 400 columns at 190°, using an argon ionization apparatus.

The technique of chromatography on silicic acid has been described by Gray and Macfarlane (16). Mallinkrodt silicic acid, 100 mesh, was employed without prewashing or oven-drying. The load ratio was approximately 1.5 mg lipid-phosphorus per g silicic acid.

RESULTS

Extraction. The pale-green heart leaves of fresh cabbage were stripped from the midrib, chopped in lots of 125 g, and immediately homogenized for 2 minutes in a Waring blendor at room temperature with 250 ml chloroform-methanol 1/1(v/v) precooled to −10°. The extract was filtered and the residue re-extracted with 1 liter solvent per kg original weight. Each extract was washed three times with an equal volume of water; this washing removes at least some of the inositide (16). After freezing out residual water, the chloroform solution was filtered, dried over calcium sulphate, and the solvent removed *in vacuo* at 40°. The residue was a dark-green oil. Two preparations were made. Preparation 1 corresponded to 0.21% total lipid and 0.093% phospholipid in fresh leaf. Preparation 2 was made in the same way with similar yield.

Purification. Preparation 1 was dissolved in 40 ml diethyl ether, and 200 ml acetone was added. After a few hours at −10°, the precipitate was collected and the supernatant was taken to dryness, dissolved in hot

acetone, and left at —10° overnight. The pooled precipitates were dissolved in ether and the phospholipids again precipitated with acetone. Recovery of phosphorus was 90%. The material (phospholipid 1) was dark brown; P, 2.4%; atomic ratio, amino-N/P, 0.36; choline-N/P, 0.34.

Preparation 2 was dissolved in petroleum ether (b.p. 40-60°) and dialyzed in a rubber glove against the same solvent for 24 hours. The nondialyzable fraction (phospholipid 2) was bright green and contained all the phosphorus; P, 2.4%; atomic ratio, amino-N/P, 0.30; choline-N/P, 0.39.

Fractionation of Total Phospholipid by Chromatography on Silicic Acid. Phospholipid 1 (4.3 g; 106 mg P) in 80 ml chloroform, was loaded on a column of silicic acid (70 g, 15 cm × 3.5 cm) prepared in chloroform. After passage of 1.2 liters chloroform, which eluted 1.1 g of fat free from phosphorus, the phospholipid was eluted with increasing concentrations of methanol in chloroform (Fig. 1). The chromatogram was completed in a total of 2.0 liters eluting solvents. The major peaks, appearing in chloroform-methanol 32/1, 9/1, and 1/1(v/v), correspond to amino- nitrogen-free, amino-N-, and choline-containing phospholipids, respectively. Eluates were pooled as shown in Figure 1 and analyzed for phosphatidic acid, amino-N, serine, ethanolamine, fatty acid ester, and unsaturation of fatty acids, as appropriate, and the component glycerolphospholipids were detected by paper chromatography of the phosphoric esters formed on mild alkaline hydrolysis.

Table 2 summarizes the results. Several of the fractions had a low phosphorus content, from which one

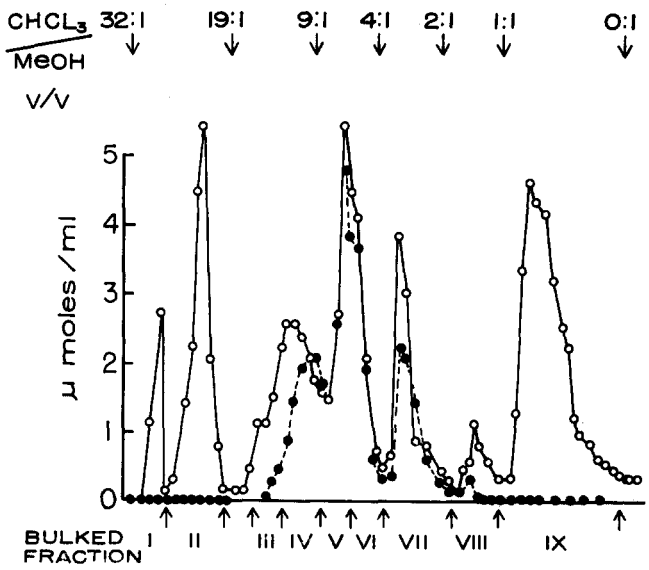

FIG. 1. Chromatography of total phospholipids of cabbage leaf (preparation 1); o = phosphorus, ● = amino-nitrogen.

may conclude the presence of a lipid impurity. This does not appreciably affect the identification of the phospholipid components but, for reasons discussed below, the unsaturation values are only approximate.

Fraction A I, which had a ratio of glycerol to phosphorus of 1.01, and gave glycerophosphate as the sole phosphoric ester on mild alkaline hydrolysis, is identified as phosphatidic acid. Fraction A II, glycerol to phosphorus ratio 1.1, contained phosphatidic acid and a phospholipid giving an unknown ester (spot D, R_f in phenol-ammonia 0.56). Fractions A I and A II were

TABLE 2. FRACTIONATION OF TOTAL PHOSPHOLIPIDS ON SILICIC ACID

Fraction	Per Cent of Total Phospholipid P	P as Per Cent of Dry Weight	Molar Ratio (P = 1.0)		Number of Double Bonds/Molecule Fatty Acids	Components Detected on Hydrolysis (Approx. Amounts as Per Cent Total P) *
			Amino-N	Fatty Acid Ester		
A I	4.3	3.2	nil	1.94	1.9	GP(95)
II	12.9	3.8	nil	1.94	0.7	GP(36); spot D
III	6.0	3.8	0.12	1.06	1.0	spot D; GPG(27); GPS(12)
IV	11.6	3.5	0.74	1.68	1.7	GPG; GPS(12); GPE(62)
V	13.0	3.5	0.87	1.58	1.7	GPG; GPS(10); GPE(77)
VI	8.0	2.5	0.83	1.97	1.1	GPS(5); GPE(78)
VII	10.0	3.1	0.64	1.82	0.5	GPE
VIII	5.2	3.0	0.26	1.50	—	—
IX	29.0	4.0	0.07	1.72	1.7	GPC

* Compounds detected in mild alkaline hydrolyzate (see Table 1); amounts computed from analysis of the fractions for phosphatidic acid, serine, ethanolamine, and glycerol.

analyzed for carbohydrate after hydrolysis in 0.5 N H_2SO_4; A I contained a trace and A II an amount equivalent to a molar ratio of glucose to phosphorus equal to 0.10, using the phenol-sulphuric acid reagent of Dubois *et al.* (20), which gives a positive test for most sugars. Fraction A III, which had a low fatty acid ester to phosphorus ratio and a glycerol to phosphorus ratio of 1.30, contained a small proportion of phosphatidylserine and two other components: the unknown present in A II (spot D) and a third component giving spot C (R_f in phenol-ammonia, 0.40). Spot C was subsequently identified (see below) as glycerophosphorylglycerol (GPG; cf. Benson and Maruo (14): R_f = 0.40). Phosphatidylglycerol was also present in fractions A IV and A V. These fractions, with A VI and A VII, constitute the cephalin fraction, containing both phosphatidylserine and phosphatidylethanolamine; the spread and consequent overlap of phosphatidylglycerol, phosphatidylserine, and phosphatidylethanolamine is probably due to the range of unsaturation of the constituent fatty acids. For example, A VII, which separated as a distinct fraction after the main cephalin peak, had a much lower iodine value. Fraction A IX gave only a glycerophosphorylcholine spot; the phospholipid was completely hydrolyzed by *Cl. welchii* lecithinase, and all the phosphorus was ester phosphorus as estimated by the method of Schmidt *et al.* (21), identifying this fraction as lecithin.

Identification of Glycerophosphorylglycerol (Spot C). Fraction A IV (34 mg) was submitted to mild alkaline hydrolysis. The hydrolyzate (71% of the original phosphorus) was concentrated to 0.10 ml, and 10 μl portions were spotted on eight lanes on acid-washed Whatman No. 3 chromatography paper (found to give no phosphorus on elution). The chromatogram was developed by the ascending method in phenol-ammonia until the solvent front was 10 inches from the origin. After evaporation of most of the phenol at room temperature, the paper was washed in acetone and dried. Areas corresponding to spot C (identified by marker lanes) were cut out and eluted by percolation with 0.1 N HCl. The combined eluates were taken to dryness at 40°, dissolved in 2.0 ml water, and analyzed. Found: total P, 4.3 μmoles; phosphomonoester, 1.4 μmoles P; glycerol, 9.0 μmoles; molar ratio glycerol/P, 2.1. The lability of GPG to acid (14) can account for the formation of some phosphomonoester.

Analysis of Spot D. Fraction A II (34.9 mg) was hydrolyzed similarly (82% recovery of P) and spot D was recovered in the same way as spot C. Found: total P, 8.9 μmoles; phosphomonoester, 0.8 μmoles; glycerol, 9.7 μmoles; molar ratio glycerol/P, 1.1. On direct oxidation with periodate, the formaldehyde produced was equal to 50% of that obtained after hydrolysis with acid and phosphomonoesterase, and there was no excess consumption of periodate. This behavior is similar to that of glycerophosphate. The ester is not a phosphomonoester, however; if it is a diester of phosphoric acid, the second substituent does not consume periodate, but has not been identified. Spot D was distinct from the polyglycerophosphate of cardiolipin (GPGPG, R_f in phenol-ammonia = 0.125).

Composition of Phospholipid 1. It was computed from the analyses that the distribution of phosphorus as per cent of the total was approximately: phosphatidic acid, 9; unknown (spot D), 11; phosphatidylglycerol, 9; phosphatidylserine, 5; phosphatidylethanolamine, 32; phosphatidylcholine, 34.

Fractionation of Phospholipids After Separation of Barium Salts Insoluble in Methanol. In the hope of improving the separation of phosphatidylglycerol by silicic-acid chromatography, the barium salts of the total phospholipids were divided into methanol-soluble and methanol-insoluble fractions and the two fractions chromatographed separately.

Phospholipid 2 was shaken in diethyl ether-methanol solution with 2% barium chloride. The barium salts (220 mg P) in 50 ml diethyl ether were treated with 4 volumes methanol; the precipitate was separated after 1.5 hours at —10°, dissolved in 20 ml ether, and reprecipitated with 150 ml methanol. The combined supernatants constituted the methanol-soluble fraction (142 mg P; atomic ratio amino-N/P, 0.27). The in-

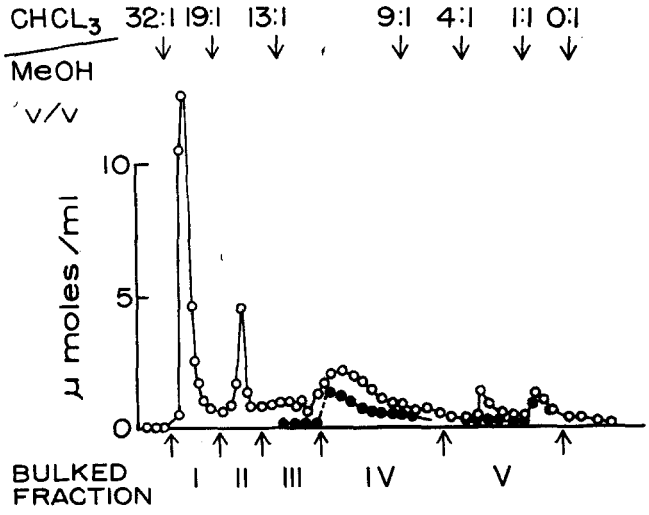

FIG. 2. Chromatography of methanol-insoluble phospholipid from preparation 2. Symbols as in Figure 1.

soluble barium salts were washed in succession with methanol, acetone, and boiling acetone and reprecipitated twice from ether solution with methanol. The combined washings contained 40 mg P; atomic ratio amino-N/P, 0.46. The barium salts were converted to the free acids by shaking the ether solution with methanol and N HCl and after washing with water, the phospholipid was dried and dissolved in chloroform (methanol-insoluble fraction, weight 1.56 g; 57 mg P; atomic ratio amino-N/P, 0.21). The methanol-insoluble material was chromatographed on 60 g silicic acid (Fig. 2 and Table 3).

The methanol-soluble fraction was taken to dryness, dissolved in diethyl ether and precipitated once with acetone. The recovered phospholipid (134 mg P) was low in phosphorus; without further purification it was chromatographed on 90 g silicic acid (Fig. 3 and Table 3).

The methanol-insoluble fraction contained, as expected, phosphatidic acid (fractions B I to B III) and phosphatidylserine; small amounts of inositide and phosphatidylethanolamine were also present. Phosphatidylglycerol and the unknown phospholipid giving spot D were both found in the methanol-soluble fraction, which also contained the lecithin and phosphatidylethanolamine. Only spot D was obtained from

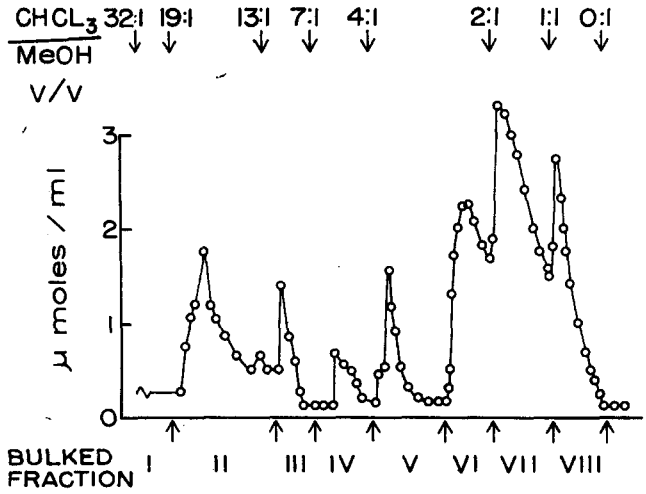

FIG. 3. Chromatography of methanol-soluble phospholipid from preparation 2. Symbols as in Figure 1.

fraction C I and the phosphatidylglycerol was contained in the large fraction eluted by chloroform-methanol 19/1(v/v), but this fraction contained a high proportion of phosphatidylethanolamine. Elution with a lower concentration of methanol in chloroform at this stage might effect separation of phosphatidylglycerol from phosphatidylethanolamine.

TABLE 3. CHROMATOGRAPHY OF METHANOL-INSOLUBLE AND METHANOL-SOLUBLE PHOSPHOLIPID FRACTIONS

Fraction	Per Cent of Total Phospholipid P	P as Per Cent of Dry Weight	Molar Ratio (P = 1.0)		Number of Double Bonds/Molecule Fatty Acids	Components Detected on Hydrolysis
			Amino-N	Fatty Acid Ester		
			Methanol-Insoluble Fraction			
B I	8.8	3.4	nil	2.30	1.6	GP
II	4.1	3.5	0.05	1.70	1.7	GP
III	2.9	5.5	0.09	0.95	—	GP; GPG (very weak)
IV	9.3	3.1	0.71	1.90	1.2	GPI; GPS; GPE
V	4.1	4.0	0.50	1.95	1.3	GPI; GPS; GPE
			Methanol-Insoluble Fraction			
C I	*	1.3	0.05	2.80	0.9	spot D
II	13.4	1.9	0.58	0.88	1.6	spot D, GPG, GPE
III	lost	—	—	—	—	—
IV	3.5	2.1	0.57	2.0	—	GPE
V	3.5	2.9	0.64	2.0	—	GPE
VI	12.0	3.9	0.02	1.9	2.4	GPC
VII	17.0	3.4	0.02	1.6	1.5	GPC
VIII	8.0	3.9	0.04	2.1	1.4	GPC
PE	4.2	2.4	1.0	2.3	0.7	—

* Partially lost.

TABLE 4. Fatty Acid Composition of Phospholipid Fractions

Fraction	Phospholipid Components	Fatty Acids as Per Cent of Total									Number of Double Bonds/Molecule	
		16–0	17–0*	18–0	16–1	18–1	18–2	18–3	Saturated	Unsaturated	Calculated	Found
B I	Phosphatidic acid	32	0	4	2	9	23	29	36	63	1.4	1.6
II	Phosphatidic acid	39	0	3	12	5	23	18	42	58	1.2	1.7
IV	Phosphatidylserine	46	2	4	trace	11	17	20	52	48	1.1	1.2
V	Phosphoinositide	45	2	5	4	4	16	19	52	43 †	1.0	1.3
C I	Unknown (spot D)	47	3	11	4	8	12	14	61	38	0.8	0.9
II	Unknown (spot D); Phosphatidylglycerol Phosphatidylethanolamine	17	0	8	5	11	31	27	25	74	1.6	1.6
A VII	Phosphatidylethanolamine	71	5	3	6	5	5	5	79	21	0.4	0.5
PE	Phosphatidylethanolamine	67	2	3	2	5	9	11	72	27	0.6	0.7
C VI	Lecithin	2	0	2	0	16	38	42	4	96	2.2	2.4
VII	Lecithin	28	0	4	trace	11	21	36	32	68	1.6	1.5
VIII	Lecithin	35	0	3	2	11	21	27	38	61	1.4	1.4
Weighted means												
B I-II	Phosphatidic acid	35	0	4	5	8	23	26	39	62		
C VI-VIII	Lecithin	21	0	3	1	13	26	36	24	76		

* Branched chain.
† Contains, in addition, 4.6%, 19–1.

Rechromatography of Fractions C IV and C V. These fractions, which gave only a GPE spot but had a low content of phosphorus, were combined, dissolved in chloroform-methanol 1/1(v/v), and washed with an equal volume of N HCl followed by water, dried, and chromatographed on 15 g silicic acid. The recovered phospholipid (60% initial P) appeared as three successive peaks when successive chloroform-methanol mixtures (9/1; 4/1; and 1/1, v/v) were applied to the column. It had an atomic ratio amino-N to phosphorus of 1.0, but the percentage of phosphorus by weight was unchanged (fraction PE, Table 3).

Fatty Acid Composition of Phospholipid Fractions. Some fractions had a rather low content of phosphorus, indicating the presence of lipid impurities. The fatty acids obtained by saponification of phospholipid fractions low in phosphorus showed a high apparent equivalent weight, ranging up to 360. No tests were made to determine the nature of this lipid impurity; the analysis of methyl esters by gas-liquid chromatography showed that it was not volatile under these conditions. In fractions B I and C I, the fatty acids recovered after saponification were noticeably less than expected from the fatty acid ester value, suggesting that the high molar ratios fatty acid ester to phosphorus were largely due to extraneous esters of short-chain, water-soluble fatty acids. In general, the molar ratio fatty acid ester to phosphorus was 2 or less, which is good reason to believe that little of the fatty acids were derived from extraneous waxes, etc.; the absence of fatty acids of chain length greater than C_{19}, shown by gas chromatography, confirms this view (22). The extent of contamination of the fractions with nonvolatile, unsaturated lipids may be judged by comparison of the unsaturation values found for the fatty acids and calculated from the gas chromatography data (Table 4).

Qualitatively, the phospholipids are of relatively simple and uniform fatty acid composition, excepting the possible occurrence of a nonadecenoic acid in either phosphoinositide or phosphatidylserine. The main saturated fatty acid is palmitic acid; the main unsaturated acids are linoleic and linolenic acids. One of the lecithin fractions (C VI) contained almost exclusively unsaturated fatty acids and it is noteworthy that the over-all unsaturation of the lecithin is greater than that of the phosphatidic acid. The high unsaturation of fraction C II fatty acids may be due to enrichment with the unsaturated fraction of the phosphatidylethanolamine.

DISCUSSION

The fatty acid composition of plant phospholipids has not previously been studied in detail. An unusual

lecithin with only palmitoleic acid was isolated from yeast by Hanahan and Jayko (23); other lecithins containing C_{18} acids were thought to be present in yeast as minor components. Palmitic acid is a major component of the inositide of wheat germ (7) and pea (24), while myristic and oleic acids were found in a preparation of soybean inositide (25). Shorland (26) found for the total phospholipids of grasses and clovers 11% palmitic acid and 76% C_{18}-unsaturated acids (approximately equal amounts of linoleic and linolenic). Shorland's results (26) are very similar to those for cabbage leaf phospholipids and point to palmitic as the characteristic saturated fatty acid and linoleic and linolenic as the characteristic unsaturated fatty acids of the leaf phospholipids. The general uniformity of fatty acid composition of the fractionated phospholipids strongly suggests that either or both of palmitic and linolenic acids are major components of the phosphatidylglycerol. This is in contrast to cardiolipin (linoleic and oleic) (16) and other phospholipids of animal tissues (27), e.g., cephalin and lecithin, which contain only traces of linolenic acid.

Phosphatidylglycerol formed a much smaller proportion of the phospholipids of cabbage leaf than was reported by Benson and Maruo (14) for the phospholipids of *Scenedesmus* cells and the leaves of tobacco and clover. For example, tobacco leaf phospholipids were found to be: lecithin, 46.5%; phosphatidylethanolamine, 7.9%; phosphatidylserine, 0.7%; phosphatidylglycerol 22%; phosphoinositide, 22.4%. These values were obtained by counting P^{32} in the individual phospholipids separated by two-dimensional paper chromatography after *in vivo* labeling; however, the specific radioactivity of the phospholipids was not determined and no account seems to have been taken of the possibility that it was not uniform.

Two phospholipids found in the cabbage leaf extracts, phosphatidic acid and the unknown giving spot D, were not detected by Benson and Maruo (14); their failure to find phosphatidic acid supports the conclusion that it is entirely an artifact formed by the action of phospholipase on other phospholipids. It is possible that the spot D phospholipid is also an artifact formed by enzyme action. The possibility that the spot D phosphoric ester is an artifact of the deacylation procedure preliminary to paper chromatography cannot be excluded in view of the formation of cyclic glycerophosphate on prolonged methanolysis of glycerophospholipids (15). However, this spot did not occur in controls, and in the hydrolyzate of fraction C I it was the only component detected on the chromatogram. The composition of the ester was not determined, though the presence of a sugar or amino-nitrogen moiety was excluded. The fact that the ester does not consume periodate in excess of a molar ratio glycerol to phosphorus of 1 is a further limitation to the number of possible residues. On the other hand, the parent phospholipid may be of the cardiolipin type, but having equimolar proportions of glycerol and phosphoric acid, a structure which would require an intramolar-ester bond.

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