Structural analysis of phosphatidylcholine of plant tissue

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ABSTRACT Pure preparations of phosphatidylcholine were isolated from spinach leaf chloroplasts, spinach leaf microsomes, and cauliflower inflorescence. The isolated phosphatidylcholine was treated with snake venom phospholipase A, and the fatty acid distribution and composition of the fatty acid methyl esters prepared from the lysophosphatidylcholine and the freed fatty acid were determined by gas–liquid chromatography. The results showed that saturated fatty acids were preferentially esterified at position 1 and unsaturated fatty acids at position 2. The phosphatidylcholine from cauliflower was also treated with phospholipase C. The resulting diglycerides were fractionated on $AgNO_3$-impregnated thin-layer plates. The diglyceride fractions were transesterified and the fatty acid composition of each was determined by gas–liquid chromatography. The predominant species contained linolenic acid only (22% of the total), linolenic and oleic acids (19%), and linolenic and palmitic acids (37%). These molecular species could not be accounted for by random distribution of the fatty acids.

SUPPLEMENTARY KEY WORDS chloroplasts · microsomes · fatty acid distribution · diglyceride species

A NUMBER of investigations have been concerned with the fatty acid composition of phosphatidylcholine in plant tissue (1–9), but relatively few investigations have described the distribution and pairing of the fatty acids in phosphatidylcholine of plants (2, 10).

In the investigations reported in this paper the fatty acid composition, distribution, and pairing of fatty acids in photosynthetic and nonphotosynthetic tissue are presented. This information was considered to be a prerequisite to subsequent studies on the mechanisms of biosynthesis of phosphatidylcholine, particularly with respect to factors that determine the final fatty acid composition. The results are compared with fatty acid analyses of phosphatidylcholine from other plant tissues and from animal tissues.

Abbreviations: BHT, butylated hydroxytoluene.

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MATERIALS AND METHODS

Extraction of Plant Tissue

Cauliflower inflorescences, obtained at local markets, were washed, grated, and then mixed with 3–4 volumes of methanol–chloroform 2:1 containing 100 mg of butylated hydroxytoluene per liter, which was added to inhibit oxidation. This mixture was homogenized at two-thirds speed in a Waring Blendor at room temperature. Extraction was by the method of Bligh and Dyer, which completely extracts lipids (11). Cellular debris was removed by filtration through cheesecloth. The filtrate was then centrifuged at 10,000 *g* for 10 min to break any emulsion that formed. The chloroform layer was drawn off and taken to dryness under vacuum, and the lipid was redissolved in about 5 ml of chloroform.

Spinach leaves, from local markets, were washed, deveined, and chopped into approximately 2-cm^2 pieces. The chopped leaves were then homogenized in a Waring Blendor at 0–4°C in 1.5 vol of 0.5 M sucrose in 0.01 M Tris–HCl, pH 8.0, for 3–4 sec. The homogenate was then filtered through four layers of cheesecloth and the filtrate was centrifuged at 200 *g* for 2 min. The pellet was discarded and chloroplasts were isolated by centrifuging the supernatant at 1000 *g* for 7 min. The chloroplasts were washed once with 10 vol of 0.5 M sucrose in 0.01 M Tris–HCl, pH 8.0. They were then resuspended in water and extracted by the method of Bligh and Dyer (11).

The 1000 *g* supernatant was centrifuged at 20,000 *g* for 30 min. The pellet was discarded and the supernatant was centrifuged at 100,000 *g* for 90 min. The resulting microsomal pellet was resuspended in water and extracted by the method of Bligh and Dyer (11). The pellets were suspended in 65 ml of water and the Bligh and Dyer extraction was started by the addition of 240 ml of CH_3OH–$CHCl_3$ 2:1.

Isolation of Phosphatidylcholine

Phosphatidylcholine was isolated from the chloroform extracts using silicic acid column chromatography (Bio-Sil-A, 100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.). The silicic acid was heated overnight at 110°C before use. The silicic acid was poured as a slurry in chloroform into a column and the column was eluted with various mixtures of chloroform and methanol. For the isolation of cauliflower phosphatidylcholine, the column dimensions were 2.5 × 80 cm. Lipid (1.5 g) was applied as a chloroform solution, and the column was eluted successively with 900 ml of $CHCl_3$–CH_3OH 9:1, 1100 ml of $CHCl_3$–CH_3OH 8:2, 800 ml of $CHCl_3$–CH_3OH 1:1, and finally, 1550 ml of CH_3OH. For the isolation of spinach phosphatidylcholine, the column dimensions were 2.5 × 60 cm. Lipid (1.0 g) was applied as a chloroform solution and the column was successively eluted with 600 ml of $CHCl_3$–CH_3OH 9:1, 800 ml of $CHCl_3$–CH_3OH 8:2, 700 ml of $CHCl_3$–CH_3OH 1:1, and finally, 1200 ml of CH_3OH. Phosphatidylcholine was eluted with the methanol. The purity of the phosphatidylcholine was checked by thin-layer chromatography, and it gave a single homogeneous spot.

For comparison, egg phosphatidylcholine (Sigma Chemical Co., St. Louis, Mo.) and the isolated samples were treated with *Crotalus adamanteus* venom (phospholipase A) (Sigma Chemical Co.) and *Clostridium welchii* phospholipase C (Calbiochem, Los Angeles, Calif.). The products of the former procedure were identified as fatty acid and lysophosphatidylcholine by thin-layer chromatography on Silica Gel G (E. Merck A.G., Darmstadt, Germany), and a product of the phospholipase C digestion was identified as 1,2-diglyceride by thin-layer chromatography. For the identification of fatty acid and diglyceride, thin-layer plates were developed in hexane–diethyl ether–acetic acid 70:30:2. To identify lysophosphatidylcholine, plates were developed in chloroform–methanol–water 70:30:4. The phosphatidylcholine also chromatographed exactly the same as egg phosphatidylcholine, using three different solvent systems with thin-layer chromatography. These were chloroform–methanol–water 70:30:4, chloroform–methanol–7 N NH_4OH 65:30:4, and chloroform–methanol–acetic acid–water 170:25:25:6 (12). The spot corresponding to egg phosphatidylcholine gave positive phosphate (13) and Dragendorff tests (14). The phosphatidylcholine isolated from the column was degraded by mild alkaline hydrolysis (15) and gave a product which migrated on paper exactly like glycerophosphorylcholine obtained from egg phosphatidylcholine (15). The spots were detected with either phosphate (16) or Schiff–periodate spray (17).

The reaction mixture for the phospholipase A digestion consisted of 200 μg of *Crotalus adamanteus* venom, 1.8 μmoles of phosphatidylcholine phosphate, 1 mg of BHT, 0.4 ml of 5 mM $CaCl_2$, and 5 ml of diethyl ether. The reaction vessel was purged with nitrogen, stoppered, and then shaken vigorously at room temperature for 3 hr. The reaction mixture for the phospholipase C digestion contained various amounts of *Clostridium welchii* enzyme in 50 mM Tris–HCl, pH 7.1, 2.5 mM $CaCl_2$, and a sixfold excess of diethyl ether. The mixture was stirred rapidly at room temperature for 3 hr. For example, 37 μmoles of phosphatidylcholine and 2 mg of BHT were suspended in the Tris–$CaCl_2$ solution (4.0 ml), 24 ml of diethyl ether was added, and the flask was flushed with nitrogen, stoppered, and then shaken for 3 hr.

Determination of Fatty Acid Composition

The methyl esters of the fatty acids of phosphatidylcholine were prepared by mixing the phospholipid with 4 ml of 5% sulfuric acid in methanol and 1 ml of benzene. 1 mg of BHT and 100 μg of methyl behenate as internal standard (Mann Research Laboratories, New York) were also added. Transesterification was carried out in a sealed tube at 70°C for 2 hr. After cooling, 1 ml of water was added and the methyl esters were extracted with pentane. The pentane was washed with water, and the samples were taken to dryness under nitrogen, redissolved in pentane, and stored at −15°C until gas–liquid chromatographic analysis.

Positional Analysis of Fatty Acids

For analysis of the positional distribution of the fatty acids of phosphatidylcholine, the phospholipid was incubated with *Crotalus adamanteus* venom (18). The 1-acyl lysophosphatidylcholine and fatty acid were separated by thin-layer chromatography, using hexane–diethyl ether–acetic acid 70:30:2 as the developing solvent. The areas on the plates corresponding to lysophosphatidylcholine and fatty acid were scraped off, and the silica gel was placed in the transesterification mixture described above. Transesterification and extraction were carried out as described.

Preparation of Diglycerides

Phosphatidylcholine from cauliflower inflorescence was

incubated with phospholipase C from *Clostridium welchii*. The incubation was carried out at room temperature with rapid stirring in 50 mM Tris–HCl, pH 7.1, and 2.5 mM with respect to $CaCl_2$ in 4.0 ml of aqueous solution with a sixfold excess of diethyl ether added. 2 mg of BHT were also added, and the flask and solvent were purged with nitrogen. The resulting diglycerides were extracted and streaked on thin-layer plates impregnated with $AgNO_3$. The plates were made by mixing 6% aqueous $AgNO_3$ with Silica Gel G in a ratio of 2.2:1. The plates were allowed to air dry in the dark and then were activated at 110°C for 2 hr. The plates were developed with chloroform–ethanol 94:6. The developed plates were then sprayed heavily with 0.01% rhodamine 6G in water, and the bands of diglyceride were visualized under ultraviolet light. The diglyceride bands were scraped, eluted, and purified to remove rhodamine by the method of Mudd, van Golde, and van Deenen (19). Aliquots of the fractionated diglycerides were again spotted on $AgNO_3$-impregnated plates to determine whether or not they gave one spot. Those that did not were rechromatographed. The purified diglycerides were transesterified, and the fatty acid methyl esters were extracted as described before.

Gas–Liquid Chromatography

The column used for gas–liquid chromatography was 10 ft × 0.25 inch copper tubing which was packed with 20% diethylene glycol succinate (Applied Science Laboratories Inc., State College, Pa.) on Chromosorb W (Applied Science Laboratories). The gas chromatograph used was a Loenco, Inc., model 160 series, instrument with flame ionization detection. The carrier gas flow rate was 60 ml/min. Column temperature was 184°C, injection temperature was 260°C, and detector, 210°C.

RESULTS

Fatty Acid Composition

Tables 1–3 show the fatty acid composition of phosphatidylcholine from spinach chloroplasts (Table 1), spinach microsomes (Table 2), and cauliflower inflorescence (Table 3). The fatty acid composition is expressed as mole % for positions 1 and 2 together, and then for positions 1 and 2 individually. The deviation from the average for duplicate determinations (two different samples used for transesterification) for undegraded phosphatidylcholine (under "Total" in Tables 1–3) was

TABLE 1 STRUCTURAL ANALYSIS OF SPINACH CHLOROPLAST PHOSPHATIDYLCHOLINE

Fatty Acid	Mole %						% Distribution			
	Total		Position 1		Position 2		Position 1		Position 2	
	a*	b*	a	b	a	b	a	b	a	b
16:0†	14.3	14.2	26.3	29.4	3.7	6.6	88	83	12	17
16:1	0.4	0.8	1.2	0.4	3.4	1.1	30	25	70	75
18:0	0.4	0.4	1.1	0.8	0.8	0.6	60	50	40	50
18:1	8.4	10.8	6.8	9.2	8.6	10.9	44	40	56	60
18:2	21.0	22.9	19.0	16.8	23.8	24.6	44	39	56	61
18:3	55.5	51.0	45.6	43.2	59.7	56.4	42	40	58	60

The phosphatidylcholine was degraded by phospholipase A_2, and fatty acid and lysophosphatidylcholine were separated by thin-layer chromatography. The methyl esters of the fatty acids synthesized by transesterification were quantitatively determined by gas chromatography. Percentage distribution was calculated using the methyl behenate internal standard.

* *a* refers to spinach harvested and analyzed in May; *b* refers to spinach harvested and analyzed in November.

† Number of carbon atoms: number of double bonds.

TABLE 2 STRUCTURAL ANALYSIS OF SPINACH MICROSOMAL PHOSPHATIDYLCHOLINE

Fatty Acid	Mole %						% Distribution			
	Total		Position 1		Position 2		Position 1		Position 2	
	a	b	a	b	a	b	a	b	a	b
16:0	20.2	23.9	38.4	46.2	6.9	7.1	85	86	15	14
16:1	0.4	0.2	2.2	0.5	1.9	0.4	55	57	45	43
18:0	0.8	2.2	2.2	0.4	0.8	0.5	71	47	29	53
18:1	12.8	18.2	12.1	12.3	15.8	16.1	44	44	56	56
18:2	25.6	25.2	17.6	21.6	25.4	41.0	41	34	59	66
18:3	40.0	31.0	27.4	18.5	49.3	34.0	36	35	64	65

Experimental procedure as in Table 1. *a* refers to spinach harvested and analyzed in May; *b* refers to spinach harvested and analyzed in November.

TABLE 3 STRUCTURAL ANALYSIS OF CAULIFLOWER INFLORESCENCE PHOSPHATIDYLCHOLINE

Fatty Acid	Mole %						% Distribution			
	Total		Position 1		Position 2		Position 1		Position 2	
	a	b	a	b	a	b	a	b	a	b
16:0	18.5	17.4	42.0	29.7	15.2	3.0	72	91	28	9
16:1		0.4		0.4		0.6		33		67
18:0	4.8	2.2	11.3	3.9	4.4	0.4	66	91	34	9
18:1	9.5	9.8	29.0	6.7	13.5	3.3	65	83	35	17
18:2	17.2	13.8	7.1	12.4	21.2	18.2	30	41	70	59
18:3	50.0	56.4	11.7	37.1	45.8	74.6	19	33	81	67

Experimental procedure as in Table 1. *a* refers to cauliflower harvested and analyzed in January; *b* refers to cauliflower harvested and analyzed in October.

less than 5%. The deviation from the average in duplicate samples for degraded phosphatidylcholine (two different samples for snake venom incubation and transesterification under "position 1" and "position 2" in Tables 1–3) was less than 7.5%. These variations do not apply to palmitoleic and stearic acids, in which percentage variation was greater as a consequence of the smaller amounts of these fatty acids. The only variations in composition that cannot be attributed to experimental error are the differences in mole % of oleic acid and α-linolenic acid (Table 2). These substantial variations in the fatty acid composition of the phosphatidylcholine could be attributable to a seasonal variation of the spinach. Analysis *a* of spinach was carried out in May and analysis *b* in November. Another possibility is that different varieties of spinach were analyzed during these two periods. Considering that the fatty acid analyses for spinach were made at different seasons, the agreement is remarkably close.

When the percentages of a fatty acid in the 1- and 2-positions are added together and divided by two, they should equal the percentage under total. However, in several cases (Tables 1–3) the values for the polyunsaturated fatty acids are lower than the number under "Total." This may be due to some oxidation of the polyunsaturated fatty acids during incubation with snake venom, although precautions were taken to prevent this by use of the antioxidant BHT and the maintenance of a nitrogen atmosphere above the samples at all times.

Fatty Acid Distribution

The last four columns of Tables 1–3 show the distribution of fatty acids between the 1- and 2-positions of glycerol of phosphatidylcholine. The general pattern for all samples of phosphatidylcholine is the predominance of saturated fatty acids in the 1-position and unsaturated fatty acids in the 2-position. The one exception is in cauliflower phosphatidylcholine (Table 3), where oleic acid is preferentially esterified at the 1-position.

Diglyceride Molecular Species

Table 4 shows the molecular species of phosphatidylcholine from cauliflower inflorescences, which were isolated and analyzed as described in Methods. The diglyceride fractions are numbered in order from the bottom to the top of the $AgNO_3$-impregnated thin-layer plate (1 to 11), i.e., in order of increasing saturation. The results in Table 4 verify the nonrandom distribution of the fatty acids indicated in Table 3. If the distribution were at all random, then diglyceride molecular species would be expected which would contain only linoleic acid, but such a species was not found. Since the same samples of phosphatidylcholine isolated from cauliflower inflorescence were used in Tables 3 (*b*) and 4, the results in these two tables can be directly compared. According to the percentages of linoleic, oleic, and palmitic acids, the percentage of the total diglycerides calculated on the basis of random distribution should be 1.9% dilinolein, 1.0% diolein, and 3.0% dipalmitin, all of which should have been detectable (see Table 4). If the distribution were random, dilinolein should have constituted 31.8% of the total diglycerides, whereas this species accounted for only 21.6%. In the case of fraction 4, random distribution predicts 19.6% of the molecular species, but the amount found was 37.2%.

From these results and calculations it is clear that neither the fatty acid distribution nor the fatty acid pairing can be accounted for by random associations.

DISCUSSION

The difference in polyunsaturated fatty acid composition between spinach chloroplasts and microsomes indicates that there is an organelle specificity for fatty acid composition, which is in contrast to the results with rat liver (20). This difference correlates with the differences in fatty acid composition of phosphatidylcholine isolated from intact spinach leaves (7, 8) and spinach chloroplast lamellae (9) (see Table 5). In the present work it should be noted that the chloroplast yield was not maximal;

TABLE 4 MOLECULAR SPECIES OF PHOSPHATIDYLCHOLINE FROM CAULIFLOWER INFLORESCENCE

Diglyceride Fraction	Double Bonds	16:0	16:1	18:0	18:1	18:2	18:3	Mole % of Each Species	Mole % Random Distribution
					mole %				
1	6	0.5			1.0	3.3	95.2	21.6	31.8
2	5	1.6			2.6	45.5	50.8	6.8	15.6
3	4	4.1	1.4		45.2		49.4	18.6	11.0
4	3	36.1		6.6	2.1	5.4	49.9	37.2	19.6
5	3	3.1	1.4		49.0	42.9	3.6	3.3	2.7
6	3 and 2	18.8	1.4	1.4	34.5	43.9		0.6	
7	2	38.8		6.4	2.4	51.4	1.2	7.5	5.4
8	2	40.9	0.4	5.3	7.1	46.3		0.7	
9	2	7.1	11.7	2.1	73.0	6.1		0.4	
10	1	40.0	15.3	5.2	36.3	3.2		1.2	
11	1	37.4	10.3	8.4	29.0	14.8		1.9	

Phosphatidylcholine was converted to diglycerides by the action of phospholipase C. The diglycerides were separated by chromatography on silver nitrate-impregnated silica gel plates. The diglycerides were transesterified in the presence of a methyl behenate internal standard. Determinations of the mole % of each diglyceride were based on the methyl behenate internal standard. Phosphatidylcholine used for these analyses was the same as that used for analysis *b* in Table 3. Random distribution was calculated by multiplying the percentage of any fatty acid by another and dividing by 50. For example the 18:2–18:3 species is 13.8 × 56.4 × 0.02 = 15.6%. In the case of species containing only one type of fatty acid, the percentage is squared and divided by 100.

TABLE 5 FATTY ACID COMPOSITION OF PHOSPHATIDYLCHOLINE FROM VARIOUS PLANT TISSUES AND SUBCELLULAR ORGANELLES

Tissue or Organelle	Fatty Acid* 14:0	16:0	16:1	16:3	18:0	18:1	18:2	18:3	20:0	Reference
Peanut		24.6	0.3		2.4	54.1	16.8	0.1	0.7	1
Soybean, 1-position†		34.4			7.6	30.2	23.8	4.0		2
Soybean, 2-position†						15.8	71.8	13.1		2
Soybean, 1- and 2-position‡		17.2			3.8	22.6	47.8	8.6		
Potato tuber		17.0			2.8	4.7	67.1	8.4		3
Apple, preclimacteric		25.2			4.2	3.8	54.1	12.8		4
Apple, postclimacteric		22.5			2.9	7.7	56.9	10.0		4
Apple mitochondria	1.5	19.3			4.9	8.9	56.3	4.2		5
Runner bean leaves		27.0			6.0	4.0	38.0	26.0		6
Spinach leaves		21.2			1.3	15.8	31.6	30.1		7
Spinach leaves		19.6	1.8§		0.8	14.2	29.3	34.5		8
Spinach chloroplast lamellae	0.3	12.4		3.6		8.7	16.3	58.2		9

* In either mole % or % by weight.
† Determinations made after treatment with phospholipase A_2.
‡ Determined by (% in 1-position + % in 2-position)/2.
§ The 16:1 fatty acid was identified as trans-3-hexadecenoic.

the 20,000 *g* pellet, which was discarded, undoubtedly contained chloroplast fragments which were thus prevented from contaminating the microsomes.

A comparison of the fatty acid composition of cauliflower phosphatidylcholine with the composition from other nonphotosynthetic tissue (Table 5) shows that cauliflower is an exception to the rule that linolenic acid content is low compared with photosynthetic tissue. But, in agreement with the composition of phosphatidylcholine, the fatty acid analysis of phosphatidylinositol from cauliflower also showed a relatively large amount of linolenic acid (21). The fatty acid analyses of cauliflower phosphatidylcholine and phosphatidylinositol dispute the idea that large amounts of linolenic acid can occur only in leaves and not in other portions of higher plants (22).

The distribution patterns of fatty acids of cauliflower and soybean phosphatidylcholine (2) agree quite closely. Also, the general pattern of distribution of fatty acids of phosphatidylcholine from the plant sources studied is similar to that of animals (22–25). There are, however, two differences from the general pattern of distribution in animals. These are the lack of asymmetry of distribution of the unsaturated fatty acids in spinach chloroplasts and microsomes and the affinity of oleic acid for the 1-position of cauliflower phosphatidylcholine. The

fatty acid distribution in plant phospholipids is probably a reflection of the amounts of different fatty acids available for phospholipid synthesis. During biosynthesis there may be a preference for placing the unsaturated fatty acid at the 2-position but no absolute specificity. When 85% of the fatty acids of spinach chloroplast phosphatidylcholine are unsaturated, clearly some unsaturated fatty acid must be at position 1.

Mudd, van Vliet, and van Deenen have prepared the diglyceride species of phospholipid isolated from spinach leaves (10). The phospholipid mixture which was degraded contained primarily phosphatidylcholine. The four diglyceride species common to both cauliflower phosphatidylcholine and spinach phospholipid are the diglycerides containing only linolenic acid, linolenic acid with linolenic acid, palmitic acid with linolenic acid, and palmitic acid with oleic acid. The spinach phospholipid did not have the diglyceride consisting of linolenic acid and oleic acid, and cauliflower phosphatidylcholine does not have the species containing only linoleic acid.

The diglyceride species of cauliflower phosphatidylcholine in general show a pattern of fatty acid pairing similar to that of animals (23–25). The pairing of polyunsaturated fatty acids with saturated fatty acids is greater than predicted by random distribution, and the pairing of polyunsaturated fatty acids with themselves or with other polyunsaturated fatty acids is less than predicted by random distribution. However, the variation from random is in no case as great as it is in the animal tissues studied.

Until the structure of biological membranes is elucidated, that is, until the orientation of lipid and protein to each other and to themselves is elucidated, the significance of the variation of fatty acid composition, distribution, and pairing cannot be determined. If membranes are indeed a bilayer structure as envisioned by Danielli and Davson (26), then except for gross variations in fatty acid composition the other variations of fatty acid distribution and pairing do not seem important. But, if proteins do bind hydrophobically to phospholipids in membranes as has been suggested by several studies (27–31), then the variation discussed could play a significant role in determining membrane structure, permeability, and stability.

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