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Phospholipids in the Developing Soybean Seed[1]

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ABSTRACT

The distribution of phospholipids in developing soybean seeds [*Glycine max* (L.) Merr., var. "Chippewa 64," "Harosoy 63," "Wayne," and "Clark 63"] was followed. From 30 to 60 days after flowering expressed as mole per cent of phospholipid phosphorus phosphatidic acid decreased from 14.8 to 9.1; phosphatidylinositol increased from 0 to 9.1; phosphatidylcholine increased from 8.2 to 9.8; phosphatidylethanolamine increased from 5.3 to 8.6; phosphatidylglycerol increased from 3.2 to 4.8; diphosphatidylglycerol increased from 2.7 to 4.1; and *N*-acylphosphatidylethanolamine decreased from 65.8 to 54.6. However, from 60 days after flowering to maturity, phosphatidic acid decreased to 0; phosphatidylinositol increased roughly 2-fold; phosphatidylcholine increased roughly 4.7-fold; phosphatidylethanolamine increased 3-fold; *N*-acylphosphatidylethanolamine decreased 11-fold; whereas phosphatidylglycerol and diphosphatidylglycerol remained essentially constant. Percentages of individual phospholipid species were not statistically different between any two varieties at a given time period.

Immature soybean cotyledons incubated with ^{14}C-acetate or -pyruvate demonstrated rapid incorporation into the phospholipid fraction. *N*-acylphosphatidylethanolamine was found to account for nearly 70% of the total radioactivity incorporated by the total polar lipid fraction and greater than 30% of the total radioactivity added.

NPE[3] has been recognized recently in higher plants from the lipids of soft wheat flour (3). Dawson *et al.* (5), and Aneja *et al.* (1) have confirmed the structure and the presence of NPE in germinating pea seeds and in a variety of plants. The amount of NPE found in plant tissue has been shown to depend on tissue age, isolation technique, and lipid extraction procedure. de la Roche and Andrews (6), using a modification of the Bligh and Dyer technique, have reported 11% NPE and 13.2% lyso-NPE from the lipids of soft wheat seed; the original paper of Bomstein (3) showed only 4% NPE in the total phospholipids extracted from wheat flour with benzene.

Singh and Privett (15, 16) have found predominately TG, PI, PE, and PC in lipid extracts from mature soybeans, and have detected NPE among the lipids from immature soybean seeds.

The objective of this paper is to describe the accumulation of phospholipids in the developing soybean seeds and to study the incorporation of radioactive acetate or pyruvate into the phospholipid fraction.

MATERIALS AND METHODS

Sampling of Plant Material. Four commercial varieties of soybeans (*Glycine max* [L.] Merr.) were used in the study reported here. "Chippewa 64", "Harosoy 63", "Wayne", and "Clark 63" were grown at the University of Illinois South Agronomy Farm, Urbana, during the 1972 growing season. A modification of the method of Rubel *et al.* (14) was used to determine DAF. Ten plants from each variety were selected at random to monitor flowering dates. At the onset of flowering, plastic tags with pencil entries indicating varietal name, date, and node were attached sequentially at intervals of 3 days to the highest flowering node of the main stem until flowering ceased. Flower opening was anticipated at dusk if petal color could be seen through the calyx.

Tagged flowers on the reference plants were used to determine seed age (DAF) at specific nodes in the remaining population. Sampling was initiated when seeds exceeded 100 mg fresh weight and continued at 10-day intervals until the seeds were yellow in color. One additional sample was taken when the seeds were fully mature. Only one pod was harvested per node from any single plant. Similar areas on the plants of all varieties were sampled. To facilitate this technique, specific groups of nodes were designated for particular picking dates, and five areas were established between the 4th and 15th node to maintain uniformity. At the proper sampling time, approximately 250 seeds were harvested during a period between 7:30 and 8:00 AM. All of the material was kept in the pod on ice and used within 1 hour of harvest. Because of the limitations in personnel and patterns of bean development, it was not possible to obtain duplicate samples at each harvest date: however, in the course of the season, replicate samples were analyzed. All of the analyses of total lipid determinations were replicated and compared by analysis of variance.

Lipid Extraction. One hundred seeds of each sample were used to follow the changes in dry weight during bean development. The remainder of the seeds were extracted for total lipids by a modification of the Bligh and Dyer (2) method or used in isotope incorporation studies. For total lipid extraction, 5-g lots of whole seed were homogenized in a Waring Blendor at 2 C for 15 sec with 100 ml of methanol, 100 ml of chloroform, and 75 ml of water. The suspension was suction-filtered and the insoluble residue was washed with an additional 40 ml of chloroform-methanol (2:1, v/v) containing 50 μg of

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[3] Abbreviations: NPE: *N*-acylphosphatidylethanolamine; TG: triglyceride; PI: phosphatidylinositol; PE: phosphatidylethanolamine; PC: phosphatidylcholine; DAF: days after flowering; PA: phosphatidic acid.

butylated hydroxytoluene per ml. The phases were allowed to separate in a 500-ml separatory funnel overnight at 2 C. The chloroform layer (lipid fraction) was then removed and reduced to approximately 2 ml under vacuum at 25 C by using a flash evaporator. The lipid materials were transferred to Teflon-lined screw cap vials with chloroform-methanol (2:1, v/v) containing butylated hydroxytoluene. The final volume was reduced to 2 ml by evaporation under nitrogen gas and stored at −15 C.

Separation of Lipid Classes. Lipid extracts were fractionated by TLC on plates coated with Absorbosil-5 (Applied Science Labs[4]) prepared in a 1:1.5 slurry with deionized water. Plates were air dried 4 hr prior to activation at 100 C for 1 hr.

Six different TLC solvent systems were used. Each system was designed to separate specific lipid classes from a total lipid extract clearly in one dimension. The systems employed are listed by increasing order of polarity: A: petroleum ether (b.p. 60–68 C)-diethyl ether-acetic acid (90:10:0.7, v/v/v); B: petroleum ether (b.p. 60–68 C)-diethyl ether-acetic acid (80:20:1, v/v/v); C: benzene-ethyl acetate-diethyl ether-acetic acid (100:10:5:0.4, v/v/v/v); D: chloroform-methanol-ammonium hydroxide (70:20:1.5, v/v/v); E: chloroform-methanol-acetic acid-water (80:15:5:2, v/v/v/v); and F: chloroform-methanol-acetic acid-water (25:15:5:2, v/v/v/v).

All chromatograms were run in equilibrated tanks when laboratory conditions were 21 to 24 C and 30 to 40% relative humidity. Lipid class identification was made by comparison of R_F values of known lipid standards and from specific reaction with iodine vapor, 0.2% 2,7-dichlorofluorescin in ethanol diluted 1:1 with water, 0.5% ninhydrin in butanol, molybdenum spray, Dittmer and Lester (7), 20% perchloric acid spray, and modified periodate Schiff's reagent, Randerath (12).

Standard NPE was prepared according to the method of Dawson *et al.* (5) by dissolving 58 mg of synthetic PE in 10 ml of chloroform, then mixing with 1.0 ml of triethylamine and 25 mg of redistilled palmitoylchloride. After 2 hr at room temperature, the mixture was shaken with 2 volumes of saturated sodium bicarbonate. The acylated product was then separated on TLC.

Preparation of Fatty Acid Methyl Esters. Polar lipid classes isolated on TLC were scraped into a 12-ml glass centrifuge tube and vortexed with 6 ml of chloroform-methanol (2:1, v/v) followed by centrifugation at 3000*g* for 5 min. The pellet was resuspended twice with 6 ml of chloroform-methanol-water (50:45:5, v/v/v), and finally with 6 ml of absolute methanol. All of the supernatant fractions were combined and reduced in volume nearly to dryness at 25 C with a flash evaporator. Fatty acid methyl esters were then prepared by the method of Craig and Murty (4). Fatty acid methyl esters were separated by GLC by using a Hewlett Packard 5750 research chromatograph fitted with a flame ionization detector. The column (2.15 m × .635 cm) was packed with 15% diethylene glycol succinate on 80:100 mesh acid-washed Chromosorb W. Column temperature was 180 C; the injection port and flame ionization detector were held at 250 C. Helium flow was maintained at 35 ml/min. Heptadecenoic acid (17:0) was used as an internal standard.

Phosphorus Determination. Phosphorus content of phospholipids separated on TLC and scraped into test tubes, was determined by Bartlett's modification of the Fiske and Subba-Row test (Parker and Peterson, Ref. 10).

[4] Mention of a trademark of proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

Incorporation of Labeled Substrate. Studies on the incorporation of radioactive acetate or pyruvate were carried out with cotyledons from "Chippewa 64" at 20, 30, and 40 DAF; and "Harosoy 63" and "Wayne" at 30, 40, and 50 DAF. Cotyledons were separated from the seed coat and were sliced uniformly with a razor blade. The slices (500 mg fresh weight) were incubated according to the procedure of Rinne and Canvin (13) with 2.5 μmoles of acetate-1-^{14}C, acetate-2-^{14}C, pyruvate-2-^{14}C, or pyruvate-3-^{14}C containing 5 μCi. Pyruvate solutions were prepared in 0.001 N H_3PO_4.

Incubations were terminated at 5, 15, 30, 60, 120, or 240 min with 5.0 ml of hot methanol. The tissue was transferred to a 40-ml TenBroeck tissue grinder with an additional 5 ml of methanol. During grinding, 10 ml of chloroform, 7.5 ml of deionized water, and 4.0 ml of chloroform-methanol (2:1, v/v with 50 μg of butylated hydroxytoluene per ml) were added. The homogenate was suction-filtered through Whatman No. 1 paper into a 50-ml glass centrifuge tube, which was subsequently spun at 3000*g* for 20 min to give two distinct phases. The chloroform phase was separated from the aqueous phase, evaporated under vacuum with 1 ml of formic acid to volatilize unmetabolized substrate, and assayed for ^{14}C in Bray's scintillator. The chloroform-soluble products were stored as stated previously. Lipid classes were separated by TLC methods previously described. Each class was scraped into a scintillation vial and counted with 10 ml of modified Bray's scintillation fluid.

Radioactivity was determined with a Packard 3003 liquid scintillation spectrophotometer. Counts per minute were corrected for quenching by the channels ratio method.

RESULTS AND DISCUSSION

Lipid class separation by TLC as described in "Materials and Methods," is shown in Table I.

The system which best resolved NPE from the total lipid contained chloroform, methanol, and ammonium hydroxide in a volume ratio of 70:20:1.5. Galactolipids were separated best by the system of chloroform-methanol-acetic acid-water (80:15:5:2, v/v/v/v). Modification of the chloroform content in the previous system from 80 to 25 volumes allowed separation of lyso-PC and lyso-PE from PI. The remaining three systems were used exclusively for neutral lipid identification.

No statistical difference was determined among the varieties by the analysis of variance for the percentage of an individual phospholipid at a given stage of development. We therefore report data only for "Harosoy 63" beans, with the exception of the information included on Tables II and IV. "Harosoy 63" seeds turned yellow at 65 DAF and were mature at 75 DAF.

Phosphorus analysis of phospholipids in immature seeds (Table III) demonstrated sizable amounts of NPE and lesser quantities of PI, PC, PE, PA, phosphatidylglycerol, and diphosphatidylglycerol in the polar lipid fraction.

Little fluctuation in the mole per cent of individual polar lipids occurred between 30 and 60 DAF, with the exception of PI which was not detected initially but increased to 9.1 mole % at 60 DAF. Greater than 50 mole % of the total phospholipid per seed was attributed to NPE in immature cotyledons.

At maturity, the mole per cent of phospholipid phosphorus and all of the phospholipid compounds except phosphatidylglycerol and diphosphatidylglycerol changed quite dramatically. These two polar lipids maintained the same mole per cent as in immature seeds. PA could not be detected at maturity. In the period from 60 DAF to maturity (approximately 15 days), PI increased from 9.1 to 17.4 mole %; PC increased

Table I. *R_F Values of Lipid Standards Determined by Thin Layer Chromatography*

All thin layer chromatograms were run in equilibrated tanks when the laboratory conditions were 21 to 24 C and 30 to 40% relative humidity. Plates were coated with 0.25 mm Absorbosil-5 and were air dried 4 hr prior to activation at 100 C for 1 hr. Solvent systems were identified in "Materials and Methods."

Compound	R_F Values in Solvent		
	A	B	C
Neutral lipid TLC			
Phospholipid	0.00	0.00	0.00
Monoglyceride	0.00	0.01	0.03
Sterol	0.06	0.09	0.24
1,2-Diglyceride	0.03	0.06	0.34
1,3-Diglyceride	0.06	0.11	0.47
Fatty alcohols	0.13	0.20	0.39
Free fatty acids	0.16	0.29	0.45
Triglyceride	0.33	0.71	0.97
Fatty acid methyl esters	0.56	0.74	0.97
Sterol esters	0.77	0.80	0.97
Hydrocarbon	0.91	0.97	0.97
	D	E	F
Polar lipid TLC			
Lysophosphatidylcholine	0.00	0.61	0.14
Phosphatidylcholine	0.21	0.23	0.50
Lysophosphatidylethanolamine	0.00	0.16	0.59
Phosphatidylinositol	0.39	0.11	0.75
Phosphatidylethanolamine	0.43	0.58	0.91
Digalactosyldiglyceride	0.26	0.37	0.91
Phosphatidylglycerol	0.60	0.31	0.91
Phosphatidic acid	0.03	0.57	0.91
Cerebroside	0.79	0.69	0.91
N-acylphosphatidylethanolamine	0.93	0.83	0.91
Diphosphatidylglycerol	0.71	0.98	0.91
Monogalactosyldiglyceride	0.83	0.98	0.91

Table II. *Average Fresh and Dry Weights During Seed Development*

Time after flowering	"Chippewa 64"		"Harosoy 63"		"Wayne"		"Clark 63"	
	Fresh wt	Dry wt	Fresh wt	Dry wt	Fresh wt	Dry wt	Fresh wt	Dry wt
days	*mg/seed*							
20	137.0	22.2	...	...	...	...	...	...
30	295.0	81.2	206.1	44.7	173.1	30.5	127.1	22.9
40	389.6	128.9	326.3	110.5	254.1	93.9	357.8	67.5
50	495.3	187.2	443.2	158.5	443.4	142.1	338.6	105.9
60	459.0	197.8	515.0	196.6	502.4	175.4	387.1	132.9
70	—[1]	...	...	...	...	...	476.2	189.9
Mature[2]	228.3	193.3	273.2	221.1	265.5	207.6	220.9	188.5

[1] No sample taken.

[2] "Chippewa 64", "Harosoy 63", "Wayne", and "Clark 63" were determined mature at 70, 75, 74, and 80 DAF, respectively.

from 9.8 to 46.0 mole %; and PE increased from 8.6 to 25.0 mole %. NPE, however, decreased from 54.6 to 4.8 mole %. Singh and Privett (15) have suggested that NPE is present in relatively large amounts in the lipid of immature soybeans; they (16) have listed the main constituents of the polar lipid fraction of "Chippewa 64" soybean (40 DAF) to be PA, PI, PE, PC, and phosphatidylserine. At maturity, they detected only PE, PC, and PI. Recently, Privett *et al.* (11) using developing soybean seeds which were frozen and stored before lipid extraction, have reported large amounts of PA and unidentified material in phospholipid fractions. Although NPE was not identified in that paper, Singh and Privett (15, 16) have previously reported large amounts of NPE in immature soybean seed. We have made preliminary studies on the effects of freezing soybean tissue before lipid extraction and have found that the concentration of NPE extracted from nonfrozen tissue was about twice the amount taken from frozen seed. In this report, lipid extracts were prepared within 1 hour after harvest from nonfrozen tissue.

The phospholipid phosphorus determination and GLC analysis of fatty acid methyl esters liberated from the whole phospholipid fraction demonstrated an increase in total phospholipid per seed over the growing season (Table IV). Therefore, the change in phospholipid distribution which occurred after 60 DAF would suggest that NPE was converted to PE and PC (Table III).

Acetate-1-^{14}C, acetate-2-^{14}C, pyruvate-2-^{14}C, and pyruvate-3-^{14}C were all incorporated rapidly into the lipid fraction. After 4 hr of incubation, 61.7% of the incorporated radioactivity from either acetate-1-^{14}C or -2-^{14}C and 61.0% of the incorporated radioactivity from either pyruvate-2-^{14}C or -3-^{14}C were in the lipid fraction. No differences were noted due to the location of the label in the substrate molecule and therefore we report only data for acetate-2-^{14}C.

Table III. *Changes in Phospholipids of Developing "Harosoy 63" Seed*

Phospholipid	DAF				
	30	40	50	60	75 (Mature)
	mole % phospholipid phosphorus/seed				
Phosphatidic acid	14.8	14.9	15.1	9.1	0.0
Phosphatidylinositol	0.0	0.4	4.4	9.1	17.4
Phosphatidylcholine	8.2	10.2	8.5	9.8	46.0
Phosphatidylethanolamine	5.3	10.3	8.5	8.6	25.0
Phosphatidylglycerol	3.2	4.0	5.8	4.8	3.6
Diphosphatidylglycerol	2.7	4.0	3.4	4.1	3.4
N-acylphosphatidylethanolamine	65.8	56.2	54.7	54.6	4.8

Table IV. *Soybean Seed Phospholipid Content*

Time after flowering	"Chippewa 64"		"Harosoy 63"		"Wayne"		"Clark 63"	
	Pi[1]	GLC[2]	Pi	GLC	Pi	GLC	Pi	GLC
days	*μm lipid phosphatide/seed*							
30	1.69	1.56	1.94	2.03	1.39	1.44	0.83	0.84
40	1.69	—[3]	1.60	2.05	1.17	...	2.28	...
50	2.30	...	2.60	2.62	1.76	...	2.05	2.10
60	2.42	...	2.44	2.29	2.39	...	2.24	...
Mature[4]	2.61	2.45	2.20	2.63	2.70	2.70	2.00	2.70

[1] Inorganic phosphorus determination.

[2] Gas liquid chromatography determination from polar lipid fatty acid methyl esters.

[3] Not analyzed.

[4] "Chippewa 64", "Harosoy 63", "Wayne", and "Clark 63" were determined mature at 70, 75, 74, and 80 DAF, respectively.

Table V. *Incorporation of Acetate-2-^{14}C by Developing "Harosoy 63" Cotyledons (30 DAF) into Lipid Classes*

Lipid Class	Radioactivity Incorporated at Various Times					
	5 min	15 min	30 min	60 min	120 min	240 min
	dpm × 10^{-2}					
Total polar lipid	621	1612	3493	6092	12,069	21,452
Monoglyceride	9	17	45	81	294	246
Diglyceride	79	300	575	999	1633	2473
Free sterol	8	11	44	60	155	354
Long chain fatty alcohol	6	14	36	94	204	666
Free fatty acid	16	25	72	99	133	186
Esterified sterol glucoside	7	9	21	44	67	176
Triglyceride	35	117	297	771	2219	6774
Sterol ester	8	27	49	107	126	243
Hydrocarbon	42	141	304	539	1144	2168

Table VI. *Incorporation of Acetate-2-^{14}C by Developing "Harosoy 63" Cotyledons (30 DAF) into Polar Lipids*

Polar Lipid	Radioactivity Incorporated at Various Times					
	5 min	15 min	30 min	60 min	120 min	240 min
	dpm × 10^{-2}					
Phosphatidylinositol	63	150	416	220	713	1238
Phosphatidylglycerol	18	47	103	154	382	466
Phosphatidylcholine	12	11	24	344	861	2239
Digalactosyldiglyceride	43	17	45	37	115	149
Phosphatidylethanolamine	9	17	33	72	244	527
Phosphatidic acid	105	243	497	375	487	949
Diphosphatidylglycerol	19	49	81	65	140	216
Monogalactosyldiglyceride	20	37	78	142	268	395
N-acylphosphatidylethanolamine	364	995	2186	4556	8723	14,449

The total polar lipid fraction had a very high incorporation percentage even though a slight decrease occurred with time (Table V). The incorporation of ^{14}C into the monoglyceride, free sterol, sterol ester, and esterified sterol glucoside classes remained stable and in combination, accounted for only 2.8 to 3.9% of the ^{14}C incorporated in the total lipids. The TG class demonstrated a slow but steady increase which reached 19.5% after 4 hr of incubation. The diglyceride class displayed a peak of incorporation between 15 and 30 min. During this period, the ^{14}C incorporation into diglycerides was twice as great as TG incorporation. The free fatty acid class gave a decreasing incorporation trend which was coincidental in magnitude to the increasing trend of the long chain fatty alcohol class. Incorporation levels of ^{14}C for the hydrocarbon class stabilized at about 6.9%. Dybing and Craig (8) reported rapid incorporation of ^{14}C from acetate into flax embryo phospholipid and 1,2-diglycerides; TG were reported to be labeled more slowly.

NPE was the predominate polar lipid to accumulate label from ^{14}C-acetate incubations (Table VI). Over the 240 min of incubation, NPE contained from 61.6% to 76.4% of the label in the polar lipid fraction, and about 30% of the total dpm added. The only other polar lipids that contained a sizeable amount of the ^{14}C were PA, PC, and PI. After 5 min, PA contained 17.7% of the label in the polar lipids; the amount decreased to 4.6% at 240 min. PC, however, showed an increase from 2.1 to 10.9% over the period of incubation. Dybing and Craig (8) reported that ^{14}C incorporation from acetate in the polar lipids was largely associated with PC in flax seed embryos and did not detect NPE. Singh and Privett (15) demonstrated an increasing relation in the incorporation of $Na_2H^{33}PO_4$ between PA and NPE of immature soybean seeds where NPE became the most highly labeled phospholipid after 24 hr.

Singh and Privett (16) have also suggested that NPE may be involved in lipid metabolism associated with maturation. Furthermore, because polar lipids are involved in TG biosynthesis (9), and both fatty acids (13) and NPE are heavily labeled from acetate-^{14}C incubations, NPE may play a role in TG biosynthesis. Studies have beeen initiated which deal with the metabolism of NPE in the developing seed, and the changes in phospholipid distribution betweeen 60 DAF and maturity.

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