Plant Physiol. (1973) 51, 468–473

Changes in Phospholipid Composition of a Winter Wheat Cultivar during Germination at 2 C and 24 C[1, 2]

Received for publication September 1, 1972

IAN A. DE LA ROCHE AND C. J. ANDREWS
Research Branch, Canada Department of Agriculture, Ottawa, Canada K1A 0C6
M. KATES
Department of Biochemistry, University of Ottawa, Ottawa, Canada K1N 6N5

ABSTRACT

Evaluation of various solvent systems for lipid extraction of wheat *Triticum aestivum* L. cv. Rideau seeds showed that boiling 2-propanol followed by the Bligh-Dyer procedure was the most efficient method, with respect to lipid yield and ability to inactivate lipolytic enzymes. Ten phospholipids were identified in dry seeds; the major components being phosphatidylcholine, lysophosphatidylcholine, N-acyl lysophosphatidylethanolamine, N-acylphosphatidylethanolamine, and phosphatidylethanolamine. After growth for 1 week (2 C) or 31 hours (24 C), the proportions of phosphatidylethanolamine + lysophosphatidic acid and phosphatidic acid increased, lysophosphatidylcholine decreased, and the remaining phospholipids showed little change. At 5 weeks (2 C) or 72 hours (24 C), the seedlings showed 5-fold increases in the proportion of phosphatidic acid largely at the expense of phosphatidylcholine, small decreases in N-acyl lysophosphatidylethanolamine and N-acylphosphatidylethanolamine, and significant increases in lysophosphatidylcholine. The changes in phosphatidic acid and phosphatidylcholine are interpreted as being partially due to increasing phospholipase D activity during germination. In general, the phospholipid composition was similar in morphologically equivalent seedlings grown at 2 C or 24 C. The increased membrane content in seedlings grown at 2 C does not reflect any preferential synthesis of individual phospholipids.

In a previous study (8) seedlings of *Triticum aestivum* L. cv. Rideau grown at 2 C were found to be more resistant to freezing temperature than their morphological equivalents germinated at 24 C. This resistance was correlated with increased synthesis and unsaturation of cell membrane phospholipids at the lower temperature. Knowledge of the changes in amounts of individual phospholipids in wheat seedlings during germination at both temperatures is required to clarify further the biochemical mechanisms underlying cold-hardiness.

The phospholipid composition of wheat seeds has not previously been investigated in detail, although considerable information is available on the phospholipid composition of wheat flour (10, 11, 19). The unusually high levels of phosphatidic acid and lysophosphatides reported for wheat flour (10, 19) suggest that significant enzymatic degradation of wheat seed lipids may occur during milling.

This communication reports on the identification and quantification of phospholipids of wheat seedlings grown under cold-hardening conditions (2 C), as compared with seedlings grown at 24 C. We have also examined various lipid extraction procedures with respect to efficiency of extraction and inactivation of degradative enzymes. The use of an extraction procedure that inactivates degradative enzymes proved to be important in distinguishing between the *in vivo* action of phospholipase D and its transphosphatidylation activity during the extraction process.

MATERIALS AND METHODS

Phospholipid Standards. Authentic preparations of phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, lysophosphatidylethanolamine, phosphatidylinositol, and phosphatidylglycerol were obtained from Calbiochem (Los Angeles) or Applied Science Laboratories (State College, Pa.). Lysophosphatidylcholine was prepared (34) from PC[3] by hydrolysis with phospholipase A (*Ophiophagus hannah*, Sigma, St. Louis, Mo.). Phosphatidic acid and lysophosphatidic acid were prepared from PC and LPC respectively, by hydrolysis with phospholipase D (Sigma, St. Louis) (16, 17). N-Acylphosphatidylethanolamine and N-acyl lysophosphatidylethanolamine were synthesized according to a modification of the Bomstein method (2) as follows: a solution of stoichiometric amounts of palmitoyl chloride and phosphatidylethanolamine (egg) in carbon tetrachloride was stirred for 72 hr at 22 C. The solvent was removed under a stream of nitrogen, and the residue was extracted by the Bligh-Dyer procedure (8). The APE was isolated from the chloroform extract by preparative TLC.

[1] Contribution No. 324 of Ottawa Research Station and No. 732 of Chemistry and Biology Research Institute, Canada Department of Agriculture.

[2] Supported in part by Grant A-5324 (M.K.) from National Research Council, Canada.

[3] Abbreviations: PE: phosphatidylethanolamine; PC: phosphatidylcholine; PS: phosphatidylserine; LPE: lysophosphatidylethanolamine; PI: phosphatidylinositol; PG: phosphatidylglycerol; LPC: lysophosphatidylcholine; PA: phosphatidic acid; LPA: lysophosphatidic acid; APE: N-acylphosphatidylethanolamine; LAPE: N-acyl lysophosphatidylethanolamine; PMe: phosphatidylmethanol; PBu: phosphatidylbutanol; GPC: glycerylphosphorylcholine; GPI: glycerylphosphorylinositol; GPS: glycerylphosphorylserine; GPE: glycerylphosphorylethanolamine; GP: glycerophosphate; GPG: glycerylphosphorylglycerol; dAPE: N-acylphosphorylethanolamine; GPMe: glycerylphosphorylmethanol.

LAPE was prepared by partial hydrolysis of APE on a neutral alumina column for 36 hr at 22 C (2). Phosphatidylmethanol and phosphatidylbutanol were produced by incubation of stoichiometric amounts of PC and methanol or 1-butanol at 22 C with phospholipase D in the presence of ether (5, 16, 35). After 6 hr, the ether was evaporated under a stream of N_2, the mixture was extracted by the Bligh-Dyer method (8), and the PMe and PBu were isolated by preparative TLC after prior removal of phosphatidic acid by precipitation of its Ca-salt from chloroform-methanol (1:10).

Chromatography. TLC was carried out on glass plates (20 × 20 cm) coated with a 0.3 mm layer of Silica Gel H (Brinkman Instruments). The TLC plates were washed first by development in chloroform-methanol (1:1, v/v) and then in acetone, air dried, activated 1 hr at 105 C and stored in a desiccator. Chromatograms were developed in the following solvent systems (given in parts by volume):

A. Chloroform-methanol-conc. ammonium hydroxide-water (70:25:3.5:2), followed by air drying and a second development in chloroform-methanol-acetic acid-water (80:10:1.5:0.7) (22).

B. Chloroform-methanol-acetic acid-water (60:8:36:4).

C. Chloroform-methanol-conc. ammonium hydroxide (70:20:2).

D. Petroleum ether (b.p. 30–60 C) diethyl ether-acetic acid (90:10:1).

The water-soluble products of alkaline and acid hydrolyses were chromatographed on Whatman No. 1 paper in water-saturated phenol-ethanol-acetic acid (50:6:5) (solvent E).

Extraction of Lipids. Fifty grams of Rideau winter wheat seed were ground in a Wiley mill and then pulverized in a ball mill for 2 min. The total lipid was extracted from triplicate 2-g lots by six different procedures:

I. Water-saturated 1-butanol; four 1-hr extractions, each with 10 ml of solvent at 22 C (19).

II. Hexane; extraction with 40 ml of solvent under reflux for 4 hr in a Goldfisch extraction apparatus (30).

III. Petroleum ether (b.p. 30–60 C); three 1-hr extractions each with 10 ml of solvent at room temperature (3).

IV. Chloroform-methanol-water (1:2:0.8); by the Bligh-Dyer procedure (8).

V. Boiling 2-propanol; two extractions each with 5 ml of solvent followed by a Bligh-Dyer extraction (8) of the meal residue; the extracts were combined.

VI. Chloroform-methanol (2:1); four 1-hr extractions with 10 ml of solvent followed by gel filtration on Sephadex G-25 (29).

All total lipid extracts were brought to dryness under N_2, and the residues were dried in a vacuum desiccator to constant weight, dissolved in 5 ml of chloroform, and aliquots were taken for phosphorus and fatty acid analysis (8).

Phospholipid Composition of Seeds. Seeds were imbibed for 5 hr at 24 C, incubated at either 24 C for 31 and 72 hr or at 2 C for 1 and 5 weeks as described elsewhere (8); 2-g lots of seeds were macerated with 0.5 g of silica gel in a mortar with pestle, and total lipids were extracted separately by the methods IV and V. An aliquot of the lipid extract, containing 20 μg of P, was streaked as a 6-cm band on a TLC plate. After development in solvent A, the phospholipids were identified by spraying one edge of the chromatogram with P-detecting reagent (9), followed by exposure of the entire chromatogram to iodine vapour. The phospholipid bands and corresponding silica gel blanks were aspirated into glass columns and eluted with 20 ml of chloroform-methanol-water (50:45:5, v/v/v) and followed by 5 ml of methanol. The extracts were taken to dryness on a rotary evaporator and lipid-P in the residues determined by a modified Bartlett procedure (14).

Fungal contamination of germinating seeds was rare; in the few cases in which it did occur, the entire Petri dish was discarded. Significant bacterial contamination did not occur, since fatty acids characteristic of bacteria (branched, cyclopropane, palmitoleic, *cis*-vaccenic,) were not detected or present only in traces (8).

Identification of Individual Phospholipid Components. Phospholipids were identified by comparing their R_F values with authentic standards in solvent systems A, B, and C. The developed chromatograms were sprayed with various visualization reagents: 0.3% ninhydrin in ethanol for amino groups; modified Dragendorff reagent (20) for identification of choline phosphatides; Schiff-periodate spray for vicinal hydroxyl groups (33), and Dittmer and Lester's reagent for the detection of phosphorus (9).

Total phospholipids were separated from the lipid extract by silicic acid column chromatography (32) and further fractioned by TLC in solvent A. Individual phospholipids were deacylated by mild alkaline hydrolysis (21), and the water-soluble products were examined by descending paper chromatography in solvent E (4).

The deacylated product of APE and LAPE (dAPE), which was soluble in both chloroform and methanol-water (10:9), was hydrolyzed in 2 N HCl in methanol for 12 min at 100 C in sealed screw-cap vials (25). The cooled hydrolysate was extracted with petroleum ether (b.p. 30–60 C), and a portion of this extract was hydrolyzed in 0.7 N methanolic-HCl for 2 hr at 110 C to cleave N-acyl groups. The water-soluble extracts of the hydrolysates were dried under a stream of nitrogen, and then over KOH in a desiccator for 12 hr. The residue was dissolved in methanol-water (10:9) and subjected to descending paper chromatography in solvent E. Petroleum ether extracts were chromatographed on TLC in either solvent C or D.

RESULTS

Extraction of Lipids. Extraction with WSB (method I; Table I) gave what appeared to be the highest yield of total lipids from wheat seed. However, the large quantity of nonlipid ma-

Table I. *Lipid Extraction of Wheat Seeds by Various Solvents*

The values were obtained from extraction of 2 g of ground Rideau wheat seed.

Method	Solvent System	Total Solvent Extracted Matter	P in Solvent Extract	Total Fatty Acid in Extract
		mg/g seed	*μg/g seed*	*mg/g seed*
I	Water-saturated 1-butanol (WSB)	38.7 ± 0.8	175 ± 3	16.0 ± 0.2
Ia	Dried WSB extract washed with Bligh-Dyer (IV)	18.8 ± 0.2	132 ± 2	13.2 ± 0.2
II	Boiling hexane	16.4 ± 0.3	13 ± 1	13.7 ± 0.2
III	Petroleum ether at room temp.	14.3 ± 0.1	18 ± 1	12.4 ± 0.1
IV	$CHCl_3$—CH_3OH—H_2O (1:2:0.8) (Bligh-Dyer)	12.0 ± 0.4	150 ± 2	6.5 ± 0.3
V	Boiling 2-propanol + Bligh-Dyer	19.7 ± 0.8	146 ± 1	12.3 ± 0.8
VI	$CHCl_3$—CH_3OH (2:1) + Sephadex G-25	21.0 ± 0.6	94 ± 1	15.4 ± .0.4

terial, including nonlipid phosphates, extracted by WSB necessitated further purification of the dried WSB extract by means of a Bligh-Dyer wash (method Ia). This resulted in decreases of 44%, 24%, and 18% in total weight, lipid-P, and fatty acid content of the extract, respectively (Table I). The presence of lipoprotein complexes in the WSB extract (13) that form a "fluff" layer at the interface of the Bligh-Dyer biphasic system (method Ia) may account for these losses.

Extraction with hot or cold petroleum ether (methods II and III) was suitable for the isolation of neutral lipids but not phospholipids, as indicated by the high fatty acid and low lipid-P contents of the extract. In contrast, the Bligh-Dyer method (IV) effectivley extracted the phospholipids but did not completely extract the neutral lipids, judging by the high lipid-P and low fatty acid contents obtained (Table I). When seeds were first extracted with boiling 2-propanol and then with the Bligh-Dyer solvent (method V), the combined extract had considerably more neutral lipids than was obtained by Bligh-Dyer solvent alone. Extraction of seeds with chloroform-methanol (2:1), followed by passage through Sephadex to remove the nonlipid contaminants, gave high total fatty acid recoveries, comparable to method I, but significantly less lipid-P than by methods I, IV, and V. Method V was therefore chosen as the most efficient procedure for quantitative recovery of lipids and also, as will be shown later, for inactivation of phospholipases.

Identification of Phospholipids. The following phospholipids were identified in the seed and the seedlings at all developmental stages (Table II): lysophosphatidylcholine, phosphatidylserine, lysophosphatidylethanolamine, phosphatidic acid, phosphatidylcholine, lysophosphatidic acid, phosphatidylethanolamine, N-acyl lysophosphatidylethanolamine, and N-acyl phosphatidylethanolamine. Phosphatidylinositol also occurred in traces in the dry seed but could not be resolved from LPC by TLC in the three solvents used (Table II). Its presence in the LPC fraction was confirmed with the identification of GPI in the deacylation products of LPC. PS and LPE could only be completely resolved in solvent B, and their identification was confirmed by detection of their deacylated, ninhydrin-positive products GPS and GPE, respectively (Table II).

Table II. *Identification of Phospholipids in Rideau Wheat by TLC Methods*

Phospholipid	$R_F \times 100$ in Solvents[1]			Reaction with Specific Stains				$R_F \times 100$ of Deacylated Water-soluble Products Solvent E[2]
	A	B	C	Ninhydrin	Choline	Schiff-HIO_4	Phosphorus	
LPC	11	11	1	−	+	−	+	88 (GPC)
PI	14	39	3	−	−	+	+	17 (GPI)
PS	16	58	3	+	−	−	+	23 (GPS)
LPE	18	36	3	+	−	−	+	64 (GPE)
PA	21	91	2	−	−	−	+	26 (GP)
PC	33	41	9	−	+	−	+	88 (GPC)
LPA[3]	40	72	30	−	−	−	+	26 (GP)
PE	40	80	24	+	−	−	+	64 (GPE)
PG	41	75	28	−	−	+	+	45 (GPG)
LAPE	53	86	43	−	−	−	+	88 (dAPE)
PMe[4]	59	95	63	−	−	−	+	57 (GPMe)
PBu[4]	61	NR[5]	NR	−	−	−	+	NR
APE	72	94	72	−	−	−	+	88 (dAPE)

[1] Solvent system A; first in $CHCl_3$:CH_3OH:NH_4OH:H_2O (70/25/3.5/2), then in $CHCl_3$:CH_3OH:acetic acid:H_2O (80/10/1.5/0.7). Solvent system B; $CHCl_3$:CH_3OH:acetic acid:H_2O (60/8/36/4). Solvent system C; $CHCl_3$:CH_3OH:NH_4OH (70/20/2). All R_F values were confirmed with authentic standards.

[2] Solvent system E; water-saturated phenol:C_2H_5OH:acetic acid (50/6/5).

[3] Values given are for cyclic LPA; noncyclic LPA was also present with $R_F \times 100$ 14 and 2 in solvents A and C, respectively.

[4] Formed by transphosphatidylation activity of phospholipase D during extraction by method IV and I respectively. Lysophosphatidylmethanol which may be formed by phospholipase D activity on LPC has R_F 0.39 in solvent A.

[5] No results.

PA migrated with the neutral lipids in the acidic TLC system B but remained at the origin in the ammonia solvent system C (Table II). The R_F values of LPA in solvents A, B, and C (Table II) suggested the presence of both cyclic and noncyclic species of LPA, which were probably formed by the action of phospholipase D (17). The water-soluble deacylation products of both LPA and PA, however, were identified as glycerophosphate (Table II). LPA and PE migrated as a single band on TLC in solvent A, but were almost completely resolved in solvents B and C; the purified LPA and PE, when deacylated, yielded GP and GPE, respectively (Table II).

PC was inseparable from digalactosyldiglyceride in all three TLC solvent systems, but was completely resolved from the glycolipid by silicic acid column chromatography using the Marinetti elution system (32); after deacylation of the isolated PC only GPC was detected. The deacylation product of both APE and LAPE (dAPE) partitioned into the chloroform as well as the methanol-water phases during workup of the deacylation mixture (see "Materials and Methods"). The dAPE was further characterized by 12-min HCl hydrolysis which yielded glycerophosphate and N-acyl ethanolamine. The N-acyl ethanolamine had an R_F 0.22 on TLC in solvent C and was completely hydrolyzed to fatty acid methyl ester and ethanolamine after 2 hr at 110 C in 0.7 N methanolic HCl.

Phosphatidyl methanol formed by transphosphatidylation activity of phospholipase D during extraction with chloroform-methanol, was identified by formation of GPMe after deacylation. However, PMe was not present when the tissues were first extracted with boiling 2-propanol. Similarly, when tissues were extracted with WSB (method I), phosphatidylbutanol was formed by the action of phospholipase D. The identity of PBu was confirmed by its mobility on TLC compared to an authentic standard; it migrated immediately above PMe in TLC solvent A (Table II).

Phosphatidyl glycerol was detected only in the lipid extracts of seedlings grown 5 weeks at 2 C or 72 hr at 24 C, respectively. This phospholipid appeared on chromatograms as a faint phosphorus-positive spot with R_F 0.41 in solvent A, identical to standard PG.

Effect of Extraction Procedure on Phospholipid Composition. Significant differences were observed in the quanities of LPC, PA, PC, and PMe recovered by the two extraction procedures, methods IV and V. Method IV resulted in considerably greater proportions of LPC in the two tissues extracted (dry and imbibed seed) as compared with method V (Table III). PA was barely detectable in the dry seed when first extracted with 2-propanol but amounted to 3% of the total phospholipid in the seed when extracted by method IV. Also, the proportion of PC was always higher in both dry and imbibed seed pre-extracted with boiling 2-propanol. PMe was not observed in the lipid extracts when treated first with boiling 2-propanol. Clearly, use of method V avoids enzymatic breakdown of phospholipids, particularly PC, during extraction and allows accurate and reliable phospholipid analyses. This procedure was used in all subsequent studies.

Table III. *Effect of Extraction Method on Phospholipid Composition*

Extractions were by the procedure of Bligh-Dyer (IV) or 2-propanol + Bligh-Dyer (V). The values are given as means ± standard error.

Phospholipid	Method IV				Method V			
	Dry seed		Imbibed (24 C)		Dry seed		Imbibed (24 C)	
	μg P/25 seed	*% P*	*μg P/25 seed*	*% P*	*μg P/25 seed*	*% P*	*μg P/25 seed*	*% P*
LPC	33.8 ± 1.0	23	29.1 ± 1.2	29	20.3 ± 1.2	14	9.4 ± 1.0	9
PA	4.6 ± 0.4	3	5.3 ± 0.5	5	Trace	Trace	4.0 ± 0.5	4
PC	57.4 ± 1.4	40	29.6 ±.1.7	30	65.8 ± 0.9	45	42.5 ± 0.3	42
PMe	1.4 ± 1.0	1	7.2 ± 0.2	7	ND[1]	ND	ND	ND
Total Lipid-P	145 ± 7.0	100	100 ± 1.6	100	145 ± 7.0	100	100 ± 1.6	100

[1] Not detected.

Table IV. *Phospholipid Composition of Lipid Extracted from Tissues*

Total lipid was first extracted with boiling 2-propanol followed by the modified Bligh and Dyer Procedure (V). The values are given as means ± standard error.

Phospholipid Component	Lipid-P					
	Dry seed	Imbibed (24 C)	31 hr (24 C)	72 hr (24 C)	1 week (2 C)	5 weeks (2 C)
	% of total					
LPC	14.0 ± 0.8	9.4 ± 1.0	5.3 ± 0.2	9.0 ± 0.5	4.9 ± 0.3	8.0 ± 0.8
PS	3.3 ± 0.2	3.1 ± 0.1	4.1 ± 0.1	4.3 ± 0.2	5.5 ± 0.3	2.2 ± 0.2
LPE	1.6 ± 0.1	2.7 ± 0.1	4.2 ± 0.1	4.7 ± 0.1	4.2 ± 0.3	2.4 ± 1.0
PA	Trace	4.0 ± 0.5	4.1 ± 0.2	20.6 ± 0.4	3.0 ± 0.2	19.6 ± 0.1
PC	45.4 ± 0.6	42.5 ± 0.3	44.6 ± 1.0	29.0 ± 0.7	41.5 ± 0.9	26.6 ± 1.1
PE, LPA	11.6 ± 1.4	12.1 ± 0.4	16.8 ± 1.0	17.2 ± 1.0	16.7 ± 0.4	22.8 ± 0.4
LAPE	13.2 ± 0.6	12.2 ± 0.8	10.2 ± 0.7	7.6 ± 0.1	12.2 ± 0.6	10.5 ± 1.0
APE	11.0 ± 0.5	14.1 ± 0.8	11.3 ± 1.0	7.4 ± 1.0	11.9 ± 0.5	7.8 ± 0

Changes in Phospholipids of Seedlings During Growth at 2 C and 24 C. Imbibing seeds for 5 hr at 24 C resulted in small but significant changes in the proportions of PA, LPE, PC and LPC as compared to the dry seed (Table IV). The over-all phospholipid composition was very similar for seedlings grown 1 week at 2 C and for 31 hr at 24 C. In both cases, the proportions of LPE and PE + LPA fractions increased while the proportion of LPC continued to decrease. The 5-week and 72-hr seedlings were also similar in phospholipid composition and showed 5-fold increases in the proportion of PA largely at the expense of PC; small decreases in LAPE and APE were also observed. The proportion of PE + LPA increased by 5% from 1 to 5 weeks at 2 C, whereas 72-hr seedlings (24 C) showed no significant change; LPC showed significant increases during corresponding periods at both temperatures.

Absolute changes in individual phospholipid content during germination at 2 C or 24 C were calculated using the percentage compositional data in Table IV and the total lipid-P values from an earlier experiment performed under identical conditions (8). The results (Fig. 1) showed that the 35% decrease in lipid-P reported earlier (8) for seeds imbibed at 24 C for 5 hr was mainly due to losses in PC and LPC. Only the fraction PE + LPA increased significantly in the first 31 hr of growth at 24 C. From 31 to 72 hr, there was a 600% increase in PA while PC decreased by 15%. The remaining fractions showed little or no change.

During the 1st week of growth at 2 C, PC increased rapidly, reaching the level found in the original dry seed (Fig. 2). Also in the 1st week, PE + LPA doubled, PS, LPE, and APE increased slightly, and PA and LPC showed no change. During the next 4 weeks at 2 C, PC, LAPE, APE, LPE, and PS remained relatively constant, but LPC, PE + LPA, and PA increased considerably in the range two to nine times the quantity found after 1 week at 2 C.

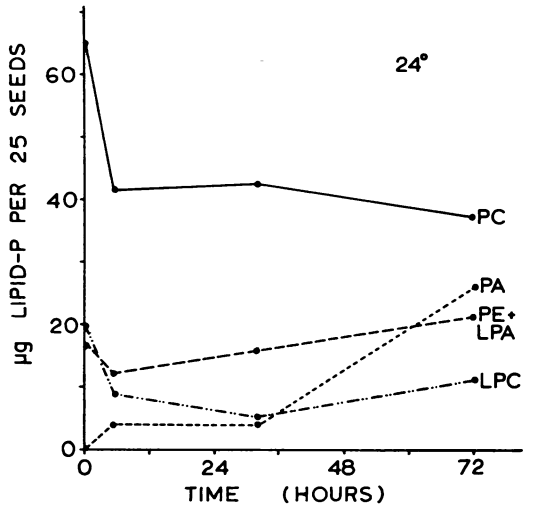

FIG. 1. Changes in phospholipid composition during growth of Rideau wheat seedlings at 24 C.

DISCUSSION

Extraction Procedures. Water-saturated butanol has been used as an extracting solvent for total lipids of commercially

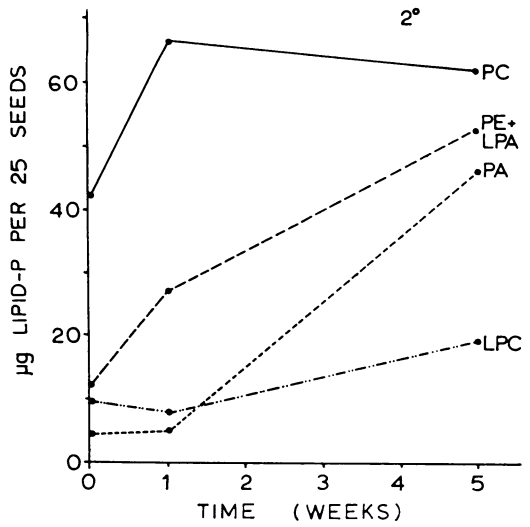

FIG. 2. Changes in phospholipid composition during growth of Rideau wheat seedlings at 2 C.

prepared wheat flour (1, 10, 19). In the present study, we found that extraction of wheat seed with WSB (method I; Table I) gave the highest yield of "lipid" material, but almost half of this proved to be nonlipid.

Although petroleum ether is used extensively as a solvent for lipid extraction of seeds rich in triglycerides (7), our results clearly demonstrate its inefficiency in extracting phospholipids (Table I, methods II and III). In contrast, the Bligh-Dyer procedure (IV) effectively extracts phospholipids but does not completely extract neutral lipids, suggesting that the chloroform-methanol-water (1:2:0.8, v/v/v) system may be too polar for complete solubilization of triglycerides. We found that extraction with boiling 2-propanol followed by the Bligh-Dyer method was the most efficient method because it resulted in high contents of both total fatty acid and total lipid-P (Table I, method IV).

Extensive lipid degradation can occur during extraction of tissues unless precautions are taken to inactivate lipolytic enzymes. Phospholipase D, found in a wide range of plant species (26), is perhaps the most important of these enzymes. As well as hydrolyzing glycerophosphatides to phosphatidic acid and the corresponding water-soluble moiety, the enzyme also possesses significant transphosphatidylase activity, being able to transfer a "phosphatidyl" unit from PC to various aliphatic alcohols, *e.g.*, methanol, butanol, glycerol, ethanolamine, ethylene glycol (5, 35). However, the structural requirements of the acceptor alcohol are fairly specific since secondary alcohols, sugars, and hydroxy acids do not react (5). Quarles and Dawson (26) showed that PMe was formed during lipid extraction of pea seeds by the Folch procedure, using a chloroform-methanol (2:1) solvent system (method VI, Table I). By using extraction methods IV and V, we found that dry wheat seeds contain a low level of phospholipase D activity (exhibited as both transphosphatidylase and hydrolase activities) which increases greatly on imbibition (Table III).

The higher proportion of LPC in the lipid extracted by method IV compared to method V suggests that enzymatic degradation of PC by phospholipase A may also take place. However, phospholipase A activity has not yet been conclusively demonstrated in seeds. The extraction studies clearly demonstrate the need to inactivate lipolytic enzymes in seed and seedlings prior to lipid extraction by Bligh-Dyer (method IV), water-saturated 1-butanol (method I), or Folch (method VI) procedures. Treating tissues with boiling 2-propanol is an efficient method for inactivation of phospholipases (15).

Phospholipid Composition. The nine phospholipids found in Rideau wheat seed and germinated seedlings, PI, LPC, LPE, PS, PA, PC, PE, LAPE, and APE, have previously been identified in wheat flour (10, 11, 19). PG was tentatively identified by Fisher *et al.* (10) in wheat flour on the basis of its deacylation product, GPG. However, its presence could not be confirmed by MacMurray and Morrison (19), and we were unable to detect any PG in dry seeds of Rideau wheat. However, we have tentatively identified it in seedlings grown in the dark for 72 hr at 24 C or 5 weeks at 2 C. This is of interest, since high proportions of PG are known to be present in leaf tissue (15).

There is considerable difference in the phospholipid composition of dry wheat seed and commercial flour preparations (10, 11, 19). The concentrations of lysophosphatides and PA are significantly higher, and PE and PC significantly lower in flour than in seed. Most reports (19) on the proportion of PA in wheat flour range from 10 to 38% of the total phospholipid. Recently, MacMurray and Morrison (19) reported that the PA content of commercial flour was negligible. It may be noteworthy that the TLC solvent system used by these authors to isolate individual phospholipids did not resolve PA and LPC. Dry wheat seeds were found to contain only trace amounts of either PA or LPA (Table IV). These results suggest that phospholipases are present and active in the original endosperm and subsequent milled flour, and are able to hydrolyze phospholipids to PA and lysophosphatides during storage. The degradative products may have important effects on the rheological properties of bread dough (23).

APE and LAPE were first identified in wheat flour by Bomstein (2) who found that these phospholipids represented 4% of the total phosphatides extracted by benzene. MacMurray and Morrison (19) subsequently showed that APE and LAPE could account for 35 to 56% of the total phospholipids in commercial flour, depending on the extraction procedure employed. We found that APE and LAPE together represented only 23% of the total phospholipid extracted from dry seed after appropriate measures had been taken to inactivate all lipolytic enzymes (Table IV).

Phospholipid Changes during Germination. Quarles and Dawson (26) reported that germination and development of pea seeds for 11 days resulted in 60% depletion of phospholipids in the cotyledons. High phospholipase D activity and a 300% increase in PMe over dry seed controls indicated that this depletion of phospholipid is primarily the result of hydrolysis due to phospholipase D (26). However, the content of PA did not increase over the 11-day period. They also showed that phospholipase D activity increased in the actively growing plumule and radicle. We obtained indirect evidence of phospholipase D activity at all stages of development of wheat at 2 C, since the PMe content of lipid extracted from 3- and 5-week-old seedlings by method IV was seven and thirteen times greater, respectively, than in the dry seed. The progressive increase in the activity of this enzyme may account for the rapid increase in phosphatidic acid during development at 2 C (Fig. 2) and at 24 C (Fig. 1). However, the fact that the decrease in PC is not sufficiently great to account for the increase in PA (Figs. 1 and 2), suggests that PA is formed *de novo* by the glycerophosphate acylase system (15).

During germination, phospholipid degradation in the endosperm (containing 88% of the total lipid-P of the dry seed) is taking place at the same time that phospholipids are being synthesized in the developing embryo (8). Unfortunately, in our study these two events were confounded because our

analyses were made on the entire seed. If we consider the indirect evidence presented above for the presence of phospholipase D in the endosperm, it is likely that there is considerable enzymatic degradation of PC in the endosperm. This decrease in PC may offset PC synthesis in the developing embryo resulting in little net change in the over-all PC content of the seedling (Figs. 1 and 2).

Increase in the PE + LPA fraction during growth at 2 C and 24 C may largely be due to increases in LPA (resulting from phospholipase D action on the lysophosphatides). This conclusion is based on a semiquantitative determination of PE at the various developmental stages.

APE has been shown to decrease rapidly in the early stages of germination in seeds of several species with the exception of oats which retains a high level of APE during the first 60 hr of germination (6). We found that the 13% content of APE in the dry wheat seed dropped significantly in the first 5 hr of imbibition and showed little change in 72 hr at 24 C (Fig. 1).

We showed previously (8) that wheat seedlings grown continuously at 2 C in the dark were capable of developing considerable cold hardiness which was associated with high levels of total phospholipid and unsaturated fatty acids. These increases are a reflection of a greater amount and a greater unsaturation of membranes synthesized during growth and adaptation of plants to low temperatures (18, 24, 28). Higher unsaturation of fatty acids in membranes increases their fluidity (28) and permeability (18) and reduces the likelihood of damage by freezing.

Increased unsaturation of membranes at low temperature could be due to changes in fatty acids *per se, e.g.*, stimulation of a desaturase; or due to preferential synthesis of particular phospholipids enriched in unsaturated fatty acids. We found that seedlings grown at 2 C had phospholipid compositions similar to the compositions of their morphological equivalents grown at 24 C (Table IV; Figs. 1 and 2). This suggests that the total phospholipid composition is the same in membranes synthesized at 2 C as it is at 24 C. Consequently, the increase in unsaturation of membranes synthesized at 2 C is likely a result of altered fatty acid desaturase activity (12) and not the result of preferential biosynthesis of individual phospholipids.

Acknowledgment—We wish to thank Dr. D. Siminovitch for useful discussion and for his review of the manuscript.

LITERATURE CITED

1. Bloksma, A. H. 1966. Extraction of flour by mixtures of butanol-1 and water. Cereal Chem. 43: 602-622.
2. Bomstein, R. A. 1965. A new class of phosphatides isolated from soft wheat flour. Biochem. Biophys. Res. Comm. 21: 49-54.
3. Brown, C. M., E. J. Weber, and C. M. Wilson. 1970. Lipid and amino acid composition of developing oats (*Avena sativa* L. cv. Brave). Crop Sci. 10: 488-491.
4. Dawson, R. M. C. 1967. Analysis of phosphatides and glycolipids by chromatography of their partial hydrolysis or alcoholysis products. *In:* G. V. Marinetti, ed., Lipid Chromatographic Analysis. Marcel Dekker Inc., New York. pp. 163-189.
5. Dawson, R. M. C. 1967. The formation of phosphatidylglycerol and other phospholipids by the transferase activity of phospholipase D. Biochem. J. 102: 205-210.
6. Dawson, R. M. C., N. Clarke, and R. H. Quarles. 1969. N-acylphosphatidylethanolamine, a phospholipid that is rapidly metabolized during the early germination of pea seeds. Biochem. J. 114: 265-270.
7. de la Roche, I. A., D. E. Alexander, and E. J. Weber. 1971. Inheritance of oleic and linoleic acids in *Zea mays* L. Crop Sci. 11: 856-859.
8. de la Roche, I. A., C. J. Andrews, M. K. Pomeroy, P. Weinberger, and M. Kates. 1972. Lipid changes in winter wheat seedlings (*Triticum aestivum* L.) at temperatures inducing cold hardiness. Can. J. Bot. 50: 2401-2409.
9. Dittmer, J. C. and R. L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin layer chromatograms. J. Lipid Res. 5: 126-127.
10. Fisher, N., M. E. Broughton, D. J. Peel, and R. Bennett. 1964. The lipids of wheat II: Lipids of flour from single wheat varieties of widely varying baking quality. J. Sci. Fd. Agric. 15: 325-341.
11. Graveland, A. 1968. Combination of thin layer chromatography and gas chromatography in the analysis on a microgram scale of lipids from wheat flour and wheat flour doughs. J. Amer. Oil Chem. Soc. 45: 834-840.
12. Harris, P. and A. T. James. 1969. The effect of low temperatures on fatty acid biosynthesis in plants. Biochem. J. 112: 325-330.
13. Hoseney, R. C., Y. Pomeranz, and K. F. Finney. 1970. Functional (bread making) and biochemical properties of wheat flour components. VII. Petroleum ether-soluble lipoproteins of wheat flour. Cereal Chem. 47: 153-160.
14. Kates, M. 1967. Paper chromatography of phosphatides and glycolipids on silica-gel impregnated filter paper. *In:* G. V. Marinetti, ed., Lipid Chromatographic Analysis. Marcel Dekker Inc., New York. pp. 1-39.
15. Kates, M. 1970. Plant phospholipids and glycolipids. Adv. Lipid Res. 8: 225-265.
16. Kates, M. and P. S. Sastry. 1969. Phospholipase D. *In:* J. M. Lowenstein, ed., Methods in Enzymology, Vol. XIV. Academic Press, New York. pp. 197-203.
17. Long, C., R. Odavic, and E. J. Sargent. 1967. The chemical nature of the products obtained by the action of cabbage-leaf phospholipase D on lysolecithin: the structure of lysolecithin. Biochem. J. 102: 221-229.
18. Lyons, J. M. and C. M. Asmundson. 1965. Solidification of unsaturated/saturated fatty acid mixtures and its relationship to chilling sensitivity in plants. J. Amer. Oil Chem. Soc. 42: 1056-1058.
19. MacMurray, T. A. and W. R. Morrison. 1970. Composition of wheat-flour lipids. J. Sci. Fd. Agr. 21: 520-528.
20. Mangold, M. K. L. 1961. Thin-layer chromatography of lipids. J. Amer. Oil Chem. Soc. 38: 708-727.
21. Marshall, M. O. and M. Kates. 1972. Biosynthesis of phosphatidylglycerol by cell-free preparations from spinach leaves. Biochim. Biophys. Acta 260: 558-570.
22. Neskovic, N. M. and D. M. Kostic. 1968. Quantitative analysis of rat liver phospholipids by a two-step thin layer chromatographic procedure. J. Chromatog. 35: 297-300.
23. Pomeranz, Y., R. D. Daftary, M. D. Shogren, R. C. Hoseney, and K. F. Finney. 1968. Changes in biochemical and bread-making properties of storage-damaged flour. J. Agr. Food Chem. 16: 92-96.
24. Pomeroy, M. K. and D. Siminovitch. 1971. Seasonal cytological changes in secondary phloem parenchyma cells in *Robinia pseudoacacia* in relation to cold hardiness. Can. J. Bot. 49: 787-795.
25. Quarles, R. H., N. Clarke, and R. M. C. Dawson. 1968. Isolation of N-acyl phosphatidylethanolamine from pea seeds. Biochem. Biophys. Res. Comm. 33: 964-968.
26. Quarles, R. H. and R. M. C. Dawson. 1969. The distribution of phospholipase D in developing and mature plants. Biochem. J. 112: 787-794.
27. Redshaw, E. S. and S. Zalik. 1968. Changes in lipids of cereal seedlings during vernalization. Can. J. Biochem. 46: 1093-1097.
28. Richardson, T. and A. L. Tappel. 1962. Swelling in fish mitochondria. J. Cell Biol. 13: 43-53.
29. Rouser, G., G. Kritchevsky, and A. Yamamoto. 1967. Column chromatographic and associated procedures for separation and determination of phosphatides and glycolipids. *In:* G. V. Marinetti, ed., Lipid Chromatographic Analysis. Marcel Dekker Inc., New York. pp. 99-162.
30. Shearer, D. A. and R. B. Carson. 1958. The determination of oil content and crude fat by the use of anhydrous ether, skellysolve B, and skellysolve F. J. Assn. Offic. Agr. Chem. 41: 414-416.
31. Tsen, C. C., T. Levi, and I. Hlynka. 1962. A rapid method for the extraction of lipids from wheat products. Cereal Chem. 39: 195-203.
32. Vorbeck, M. L. and G. V. Marinetti. 1965. Separation of glycosyl diglycerides from phosphatides using silicic acid column chromatography. J. Lipid Res. 6: 3-6.
33. Waldi, D. 1965. Spray reagents for thin-layer chromatography. *In:* E. Stahl, ed., Thin-layer Chromatography. A Laboratory Handbook. Academic Press, New York. pp. 483-502.
34. Weber, E. J., I. A. de la Roche, and D. E. Alexander. 1971. Stereospecific analysis of maize triglycerides. Lipids 6: 525-530.
35. Yang, S. F., S. Freer, and A. A. Benson. 1967. Transphosphatidylation by phospholipase D. J. Biol. Chem. 242: 477-484.