COMMUNICATIONS

Characterization of the Phospholipid Composition of Wheat Roots Using High-Performance Liquid Chromatography

The phospholipids of wheat roots (*Triticum aestivum* L. var. Arthur) were separated by high-performance liquid chromatography into fractions eluting in the order phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, a fraction containing phosphatidic acid and phosphatidylserine, and phosphatidylcholine. Phosphatidylcholine and phosphatidylethanolamine were the predominant phospholipids, accounting for over 85% of the total lipid phosphorus. The fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine were nearly identical, indicating that biosynthetic reactions subsequent to phosphatidic acid are not selective in regard to acyl chain composition.

Phospholipids are important components of biological membranes. Membrane properties are influenced by both the nature of the polar head groups and the acyl chain. In our work on the role of phospholipids in the structure and function of plant membranes, we required a method which permitted rapid separation of individual phospholipid classes, which permitted the quantitation of these classes, and which yielded enough lipid for analysis of the fatty acid composition of individual phospholipid classes. A large number of solvents and stationary phases exist for the separation of plant phospholipids using both one- and two-dimensional thin-layer chromatography (TLC) with the methods of Khan and Williams (1977) and Parsons and Price (1979) serving as representative examples. Two-dimensional TLC provides the best separation; however, it is time consuming, is limited by the amount of lipid that can be applied to a plate, and can result in the oxidation of polyunsaturated lipids. The oxidation of polyunsaturated lipid tends to occur during plate spotting, between successive developments, and during drying (Hitchcock and Nichols, 1971). The purpose of our study was to characterize the phospholipids of wheat (*Triticum aestivum* L. var. Arthur) roots by using high-performance liquid chromatography (HPLC). HPLC separation of the phospholipids of vegetative tissues of higher plants has not been reported previously.

MATERIALS AND METHODS

Wheat (*T. aestivum* L. var. Arthur) seedlings were grown for 5 days at 25 °C in paper seed towels. Five grams of roots was harvested for phospholipid extraction. Roots were enclosed in an aluminum foil envelope and placed at 100 °C for 15 min to inactivate phospholipases (Roughan et al., 1978). The roots were then frozen in dry ice and ground by using a mortar and pestle. A Folch extraction was performed on the dry ice powder (Folch et al., 1957). The lipid sample was filtered through a 0.4 μm pore size polycarbonate membrane (Bio-Rad Laboratories, Richmond, CA); the solvent was evaporated with a stream of nitrogen gas, and the sample was dissolved in 0.5 mL of chloroform. An aliquot containing ~1 mg of phospholipid was injected into the HPLC.

Liquid chromatographic analysis was performed with a Waters' Associates liquid chromatograph (Waters' Associates, Milford, MA). Two Model 6000 A solvent pumps were used in conjunction with a Model 660 solvent programmer for gradient elution. The chromatographic system also included a U6K injector and a Model 450 variable-wavelength detector. A 30 cm × 3.9 mm Porasil column (Waters' Associates) packed with 10 μm diameter silica gel particles was used.

The solvent system selected was a mixture of hexane, 2-propanol, and water. Hexane and propanol were commercially distilled in glass (Burdick and Jackson Laboratory, Inc., Muskegon, MI). Water was distilled, deionized, and filtered through a 0.45-μm Millipore filter before use. All solvents were ultrasonicated for 5 min prior to use to minimize dissolved gases. Solvent composition was initially hexane–2-propanol–water (6:8:0.5 v/v/v). A linear increase in the water content during a 25-min period resulted in a final solvent composition of 6:8:1.5. Solvent flow was maintained at 1.5 mL/min. Detection of eluting components was by absorbance at 206 nm.

Phospholipid standards (Supelco, Inc., Bellefonte, PA) were used to identify the components of wheat root lipids purified via high-performance liquid chromatography. Aliquots of collected fractions were spotted on silica gel thin-layer plates and developed with either a "basic" solvent system, chloroform–methanol–ammonium hydroxide–water (70:25:3:2 v/v/v/v), or an "acidic" solvent system, chloroform–methanol–acetic acid–water (65:15:10:4 v/v/v/v). Spots on thin-layer plates were visualized with iodine vapor. The phosphorus content of the fractions collected from the HPLC was determined by using Bartlett's modification (Bartlett, 1959) of the Fiske–SubbaRow method (Fiske and SubbaRow, 1925). The fatty acid composition of the phospholipid fractions was determined by gas chromatography as previously described (Hilton et al., 1971).

RESULTS

Lipids in the phospholipid class from wheat roots were separated into five fractions by using HPLC (Figure 1). Separation was achieved within 30 min. Even with minimum sample preparation, background absorbance was low and base-line stability was excellent. The five fractions cochromatographed with authentic standards (Figure 2) and were identified as phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), a fraction containing phosphatidic acid (PA) and phosphatidylserine (PS), and phosphatidylcholine (PC).

Aliquots of the phospholipid fractions collected as they eluted from the HPLC column also cochromatographed with authentic phospholipid standards on silica gel TLC plates in two different solvent systems. Comparison of R_f values (data not presented) facilitated fraction identifi-

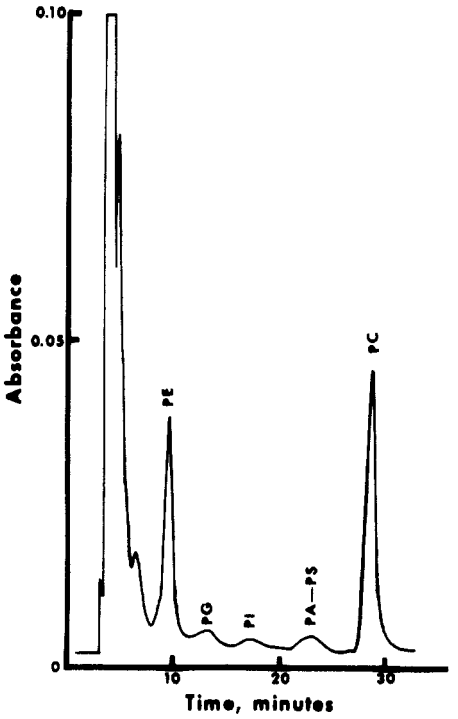

Figure 1. Separation of phospholipids isolated from wheat roots using HPLC. Chromatographic conditions: Porasil column; hexane–2-propanol–water solvent system; 25-min gradient from 6:8:0.5 to 6:8:1.5 (v/v/v); 1.5 mL/min; detection at 206 nm. Peaks were identified as PE (phosphatidylethanolamine), PG (phosphatidylglycerol), PI (phosphatidylinositol), PA–PS (phosphatidic acid–phosphatidylserine), and PC (phosphatidylcholine).

Table I. Phospholipid Composition of Wheat Seedling Roots[a]

phospholipid fraction	phospholipid/root fresh wt, μg/g	total lipid phosphorus, %
phosphatidylethanolamine	287	35
phosphatidylglycerol	36	4
phosphtidylinositol	52	6
phosphatidic acid–phosphatidylserine	31	4
phosphatidylcholine	421	51

[a] Phospholipid fractions were separated by using high-performance liquid chromatography. Fractions were collected and the phosphorus content was determined by Bartlett's modification of the Fiske-SubbaRow method. Phospholipid = amount of lipid phosphorus × 25.

cation; however, neither thin-layer system provided adequate resolution of all components.

Quantitation of the HPLC fractions by phosphorous analysis revealed that the predominant phospholipids isolated from wheat seedling roots were PC and PE (Table I). Half of the lipid phosphorous was in PC and one-third was in PE. PG, PA, PS, and PI were isolated in much smaller quantities.

The fatty acid composition of the HPLC fractions was determined by GLC (Table II). The fatty acid compositions of PE and PC were nearly identical. Palmitate, linoleate, and linolenate accounted for 30, 48, and 15%, respectively, of the fatty acids esterified to PE and PC from wheat roots grown at 25 °C. Stearate and oleate were present in smaller quantities.

DISCUSSION

Our HPLC procedure resolved the phospholipid classes of wheat roots within 30 min (Figure 1). Sufficient

Table II. Fatty Acid Composition of Phosphatidylethanolamine and Phosphatidylcholine Isolated from Wheat Seedling Roots[a]

phospholipid fraction	% by wt for fatty acid composition				
	16[b]	18	18:1	18:2	18:3
phosphatidyl-ethanolamine	32	3	4	47	14
phosphatidylcholine	27	2	5	49	16

[a] Phospholipids were separated by using high-performance liquid chromatography. The phosphatidylethanolamine and phosphatidylcholine fractions were collected and the fatty acid composition was determined. [b] 16 = palmitic acid, 18 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, and 18:3 = linolenic acid.

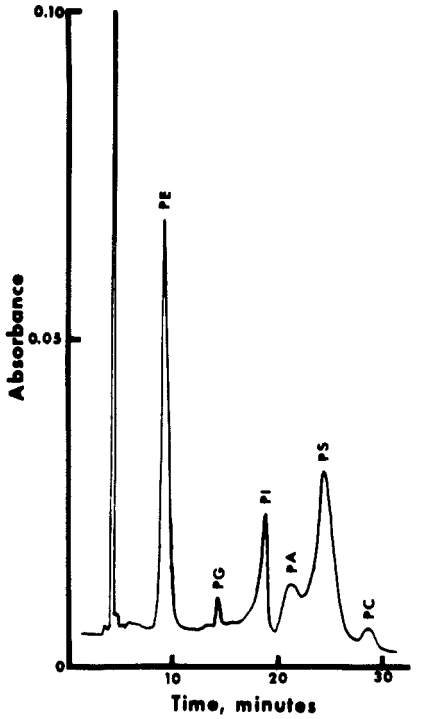

Figure 2. Separation of phospholipid standards by HPLC. Chromatographic conditions: Porasil column; hexane–2-propanol–water solvent system; 25-min gradient from 6:8:0.5 to 6:8:1.5 (v/v/v); 1.5 mL/min; detection at 206 nm. Peaks were identified as PE (phosphatidylethanolamine), PG (phosphatidylglygerol), PI (phosphatidylinositol), PA (phosphatidic acid), PS (phosphatidylserine), and PC (phosphatidylcholine).

quantities of the phospholipids were separated to allow for chemical analysis (Tables I and II). Also, the closed chromatographic system minimized the probability of oxidation of highly unsaturated fatty acids. Even though HPLC offers several advantages over TLC, none of the published HPLC techniques for separation of complex lipids met all of our objectives. Erdahl et al. (1973) and Privett et al. (1973) described the lipid composition of developing soybean colytedons based on HPLC. Their method provided excellent resolution of the phospholipids and other lipid species. However, the separation required 2.5 h and two complex solvent systems.

Geurts Van Kessel et al. (1977) and Hax and Geurts Van Kessel (1977) reported rapid separation (40 min) and good resolution of the phospholipids of red blood cell ghosts and squid axions, respectively. We have modified and simplified their solvent system (hexane, propanol, and water) and solvent program and decreased the separation time. Their initial solvent composition was 6:8:0.75 (v/v/v) hexane–propanol–water; final composition was 6:8:1.4. The

solvent program, at a flow rate of 1 mL/minute, was 5 min isocratic, 15 min linear increase in water from 0.5 to 1.4, and 20 min isocratic at the final solvent composition of 6:8:1.4. We achieved excellent separation (Figures 1 and 2) in 10 min less time beginning with a solvent composition of 6:8:0.5 and running a linear increase in water to a final composition of 6:8:1.5, flow rate 1.5 mL/min.

The methods for phospholipids of red blood cell ghosts and squid axions did not separate sufficient quantities for further analysis. The data in Table I show that our method separates sufficient quantities of the phospholipids for quantitation by colorimetric phosphorus determination. The data also confirm the predominance of PC and PE in wheat root membranes observed by Willemot (1975). Willemot's results are based on incorporation of radioactive phosphorus into phospholipids separated by TLC.

The fatty acid compositions of PE and PC were nearly identical (Table II). Phospholipids are synthesized via a common intermediate, PA. PC and PE can be synthesized by condensation of digylceride, derived from PA, with either CDP-ethanolamine or CDP-choline (Kates and Marshall, 1975). Some evidence suggests that both these condensation reactions are catalyzed by the same enzyme. PC can also arise from stepwise methylation of PE. Our data suggest that in wheat roots, biosynthetic reactions for PC and PE subsequent to PA are not selective in regard to acyl chain composition.

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Tritium Labeling of Avermectins B_1a and B_2a via Stereoselective Reduction of 5-Ketoavermectins

The potent parasiticides avermectins B_1a and B_2a have been labeled in the 5 position by stereoselective reduction of the corresponding 5-ketoavermectins using sodium borotritide. The 5-ketoavermectins were prepared in 40–55% yields by manganese dioxide oxidation of the natural products.

Recent reports from these laboratories have described the discovery (Burg et al., 1979), isolation (Miller et al., 1979), and structure determination (Albers-Schönberg, et al., 1981; Springer et al., 1981) of the avermectins, a new family of complex macrocyclic lactones elaborated by the soil actinomycete *Streptomyces avermitilis*. The avermectins (Figure 1) exhibit potent activity against a wide variety of parasitic helminths (Egerton et al., 1979; Blair and Campbell, 1979a,b; Campbell and Blair, 1979), arachnids (Centurier and Barth, 1980), and insects (Ostlind et al., 1979; James et al., 1980), apparently by selective blockade of γ-aminobutyric acid mediated neurotransmission (Fritz et al., 1979; Pong et al., 1980; Pong and Wang, 1980; Kass et al., 1980). Such activity is unprecedented among macrocyclic lactones (Burg et al., 1979; Masamune et al., 1977; Zahner, 1977). The further study of the mode of action of the avermectins, as well as metabolic, pharmacokinetic, and tissue residue analyses, required radiolabeled materials, and we wish to report the preparation of 5-[^{3}H]avermectins B_1a (1) and B_2a (2) via redox cycling through the 5-ketoavermectins **3** and **4**, respectively, a convenient method which should be applicable to a variety of avermectin derivatives.

EXPERIMENTAL SECTION

Materials. Avermectins B_1a (**5**) and B_2a (**6**) were supplied by the Natural Products Isolation Department of the Merck Sharp & Dohme Research Laboratories and were >99% pure. Activated manganese dioxide (Winthrop Laboratories) and sodium [^{3}H]borohydride (New England Nuclear Corp.) were used as received. All solvents were of high-performance liquid chromatography (HPLC) or

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