Characterization of the Phospholipids in *Pinus ponderosa* Pollen*

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ABSTRACT: Studies on the phospholipids extracted from *Ponderosa* pine pollen have established the presence of the following: phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmyoinositol, phosphatidylserine, and bisphosphatidylglycerol.

Identification of these lipids was based on group analysis of each component produced by acid and basic hydrolysis, and on chromatographic comparison of each intact phospholipid with known standards. Palmitic, oleic, and linoleic acids are the major fatty acids of each phospholipid.

Interest in the chemical composition of pollen grains is growing rapidly, as illustrated by recent publications on pollen polysaccharides (Bouveng, 1963, 1965), nucleic acids (Tano and Takahashi, 1964; Stanley and Young, 1962), and pigments (Strohl and Seibel, 1965). Previously, work on the chemistry of pollen had been confined mainly to the allergenic proteins. Biochemical studies of the changes occurring in the germinating pollen grain during the fertilization process have also become intensified (Stanley and Loewus, 1964).

As yet, little is known about the lipids of pollen, although several laboratories have investigated pollen waxes (Nilsson, 1956; Nilsson *et al.*, 1957), while Scott and Strohl (1962) have described some of the lipids of pine pollen. The latter workers identified wax esters and free fatty acids and alcohols which are associated with the rigid outer spore coat of the grain, and confirmed the abundance of triglycerides within the pollen cell. In their work all hydrocarbon derivatives were found to be straight-chain molecules, most of them containing an even number of carbon atoms, from 16 to 30. Very recently, Shaw and Yeadon (1966) reported on the lipids of the exine, the outer wall of the pollen.

Apparently no detailed study has been made of pollen phospholipids. This paper reports the isolation and identification of the saponifiable phospholipids found in *Pinus ponderosa* pollen, and was carried out as a preliminary to the investigation of changes which occur in the phospholipids during pollen germination.

Experimental Procedure

Materials. Ponderosa pine pollen was collected in May 1964 at the Institute of Forest Genetics, U. S. Department of Agriculture, Placerville, Calif., with the assistance of Drs. R. M. Echols and Robert G. Stanley. The mature catkins were air-dried indoors, and the pollen which was liberated from them was filtered free of its woody contaminants and stored at −10°.

Glycerylphosphorylcholine was prepared by mild alkaline hydrolysis of purified lecithin, a gift of Dr. H. S. Hendrickson. Phosphatidylglycerol from *Azotobacter agilis* was a gift of Dr. John Law. All other intact and deacylated phospholipid standards were obtained from ox brain or *Mycobacterium phlei.*

Glycerylphosphorylmethanol was prepared by esterification of glycerol 3-phosphate, as its cyclohexylamine half-salt, with freshly prepared diazomethane (Arndt, 1957). Glycerol 1,2-phosphate was made from glycerol 2-phosphate, according to Brown *et al.* (1958).

1-(Glycerol-3′-phosphoryl)glycerol 3-phosphate was made from 1,3-di-*O*-(L-glycerol-3′-phosphoryl)glycerol, isolated from beef heart cardiolipin (LeCocq and Ballou, 1964), by reaction with 0.5 molar equiv of sodium metaperiodate and subsequent removal of the glycolaldehyde with 1,1-dimethylhydrazine (Brown and Stewart, 1964). The desired product was purified by paper chromatography and gave the expected ratios of glycerol, phosphorus, and α-linked glycerol. Fatty acid methyl ester mixtures H-104 and L-205 were obtained from Kensington Scientific Corp.

Methods. Phosphorus was determined by the method of Bartlett (1959); total and α-linked glycerol, by the technique of Hanahan and Olley (1958); myoinositol, according to the microbiological assay using *Kloeckera brevis* (Snell, 1950); choline, as reported by Wheeldon and Collins (1958); and total nitrogen, by the method of Long and Staples (1961).

Extraction and Deacylation of Lipids. Advantage was taken of the swelling of pollen grains in chloroform–methanol mixtures to achieve both fracturing and extraction in a single step. Air-dried pollen (100 g) was extracted with chloroform–methanol (2:1, v/v) in three successive portions of 750 ml each, while the pollen was being fractured in a rotating porcelain ball mill. The operation was carried out at 4° for a total of 3 hr, and the progress of pollen breakage was followed microscopically. Efficiency of extraction was

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monitored by organic phosphorus analysis, the three successive extracts containing, respectively, 82, 9.5, and 0.3 mg of lipid phosphorus. The extracts were combined and washed with two-tenths volumes of 0.05 N sodium chloride solution (Folch *et al.*, 1957), and the aqueous phase was discarded. The interfacial solid phase was isolated and saved for further extraction. The organic solution was reduced in volume on a rotary evaporator at 35° prior to deacylation.

The interfacial solid was refluxed in acetone for 2 min, and the filtered dried residue was washed with 95% ethanol and then extracted twice with 100 ml of chloroform–methanol–concentrated hydrochloric acid (100:50:0.2). The combined chloroform–methanol extracts were washed with two-tenths volumes of 1 N hydrochloric acid, followed by two-tenths volumes of water, and pooled with the larger lipid extract isolated by neutral chloroform–methanol extraction.

In a subsequent preparation, air-dried pollen was extracted with chloroform–methanol–concentrated hydrochloric acid (200:100:1). This acidified extractant removed an additional 10% of lipid phosphorus from the pollen over that extracted with neutral chloroform–methanol, although there was no obvious difference in the composition of the product.

Deacylation of the lipids was performed as described earlier (Ballou *et al.*, 1963), with slight modification. The methanol:chloroform ratio was increased to 1:1, in order to minimize destruction of the hydrophilic portion of the phospholipid (Brockerhoff, 1963); and ammonium, rather than cyclohexylamine salts of the deacylated phospholipids were prepared. Deacylated lipids were stored in salt form at −10°.

Column Chromatography of the Deacylated Phospholipids. Initial separations were achieved by anion-exchange chromatography on DEAE-Sephadex (carbonate form). A 4-ml aqueous solution of the deacylated products was placed on a column of DEAE-Sephadex (1.7 × 40 cm), and elution was carried out with a linear gradient of ammonium bicarbonate, pH 7.5. A gradient (1 l.) from 0 to 0.2 M ammonium bicarbonate was collected in 4-ml fractions, and the elution pattern was described by the phosphorus content of every other fraction. The appropriate fractions were then combined and evaporated to dryness several times on a rotary evaporator at 35° to remove most of the ammonium bicarbonate.

Analytical Paper Chromatography. The deacylated phospholipids, their hydrolysis products, and the synthetic and deacylated standards were separated by descending chromatography on citrate-washed Whatman No. 1 paper. A basic solvent system A, isopropyl alcohol–ammonia–water (7:1:2), and an acidic system B, isopropyl alcohol–formic acid–water (80:13:7), were used with the deacylated lipids. 1-Butanol–acetic acid–water (4:1:5) and phenol–water (100:38) were also employed and are referred to as solvent mixtures C and D, respectively.

Phosphorus-containing compounds on paper were detected by the Hanes–Isherwood spray (Hanes and Isherwood, 1949). Ninhydrin spray was used for amino nitrogen detection, a modified Dragendorff dip for quaternary ammonium groups (Bregoff *et al.*, 1953), and the silver nitrate (Trevelyan *et al.*, 1950) and periodate–benzidine (Gordon *et al.*, 1956) procedures for detecting vicinal hydroxyl groups and α-amino alcohols.

Preparative Paper Chromatography. Deacylated phospholipids, from the initial DEAE-Sephadex column separation, were isolated and purified by paper chromatography on Whatman No. 3 paper, using either solvent system A or B. The compounds were eluted from paper with distilled water and were freed of paper contaminants by placing the samples on 1.3 × 8 cm columns of DEAE-Sephadex (carbonate), washing liberally with water, and finally eluting with 0.4 M ammonium carbonate. This purification procedure was essential for accurate glycerol analyses on these preparations.

Paper Electrophoresis. Deacylated phospholipids were separated by zone electrophoresis on Whatman No. 3 paper, 6 × 22.5 cm, using an apparatus similar to that described by Crestfield and Allen (1955). Pyridine acetate buffer at pH 6.0 was used, with applied voltages of 750–850 v for 1–2 hr.

Thin Layer Chromatography. The intact phospholipids were chromatographed in one direction on silica gel G or H of 0.25–0.50 mm thickness. Chloroform–methanol–water (65:25:4) was used for the phosphatides (Wagner *et al.*, 1961) and benzene for fatty acid methyl esters and other methylated hydrocarbon derivatives (Morrison and Smith, 1964). Chloroform–methanol–acetic acid–water (60:30:8:4) was used for preparative separations of phosphatidylmyoinositol and phosphatidylserine on silica gel H prepared with 10 mM sodium carbonate (Skipski *et al.*, 1962).

The "molybdenum blue" spray of Dittmer and Lester (1964) was used for detecting phosphate-containing lipids on thin layer plates. Ninhydrin spray and a 50% sulfuric acid spray, followed by heating at 120°, were used where applicable. Iodine vapor detection was employed for initial survey of the plates. In those cases where the fatty acids were to be studied, a Rhodamine 6G spray (0.5% ethanolic solution) was used, and lipids were detected by their fluorescence under ultraviolet light. Quantitative data on the intact phospholipids were obtained by eluting each phosphatide from the thin layer plate and analyzing for phosphorus, with correction against a "blank" sample from the same plate.

Preparation of Purified Intact Phospholipids. Air-dried pollen (30 g) was extracted three times with 300-ml portions of chloroform–methanol–concentrated hydrochloric acid (200:100:0.2) at 4° for a total of 3 hr. After the saline wash, a crude phospholipid fraction was obtained by the silicic acid slurry technique of Abramson and Blecher (1965). This material was streaked along the origin of five thin layer plates of 0.5 mm thickness, and the plates were developed with chloroform–methanol–water (65:25:4). Rhodamine 6G was used for detection of the lipids, with the aid of guide strips sprayed with "molybdenum blue." The

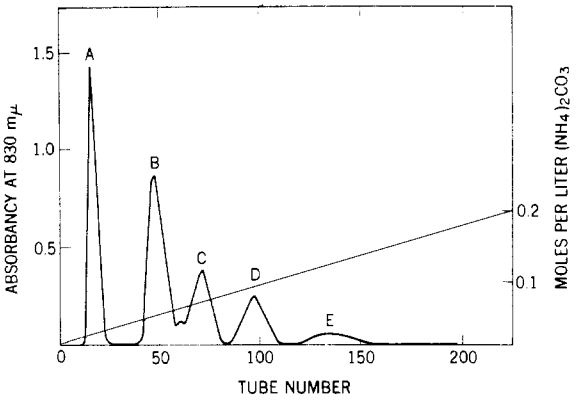

FIGURE 1: Gradient elution pattern of the deacylated phospholipids from DEAE-Sephadex (carbonate). Peaks are described by the total phosphate content of the fractions.

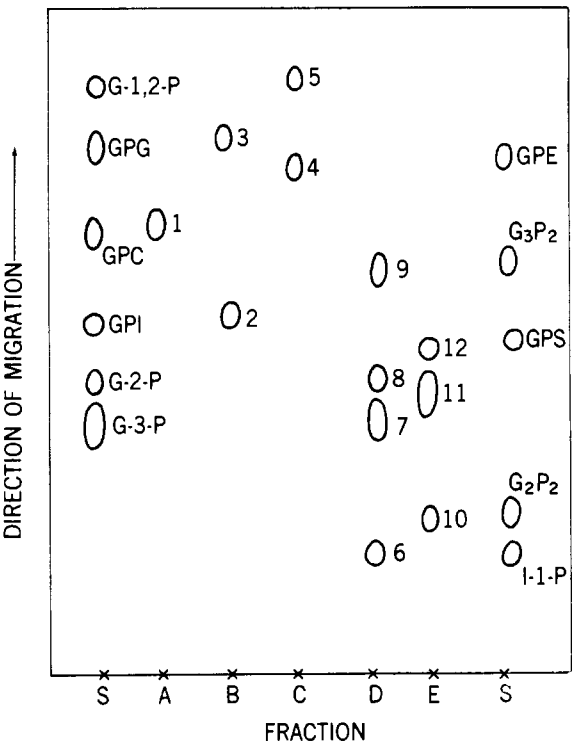

FIGURE 2: Paper chromatogram of the deacylated phospholipids in peaks A through E obtained by DEAE-Sephadex column chromatography (Figure 1). The phosphate-containing spots, separated by development with isopropyl alcohol–ammonia–water (7:1:2), are shown for each peak. S denotes the reference compounds used. G-3-P, glycerol-3-phosphate; G-2-P, glycerol-2-phosphate; GPI, glycerylphosphorylmyoinositol; GPC, glycerylphosphorylcholine; GPG, glycerylphosphorylglycerol; G-1,2-P, glycerol-1,2-phosphate, I-1-P, myoinositol-1-phosphate; G_2P_2, 1-(glycerol-3′-phosphoryl)glycerol 3-phosphate; GPS, glycerylphosphorylserine; G_3P_2, 1,3-di-*O*-(glycerol-3′-phosphoryl)glycerol; GPE, glycerylphosphorylethanolamine.

bands were scraped from the plates and the phospholipids were eluted with absolute methanol. The process was repeated until each lipid gave a single spot on thin layer chromatograms. Phosphatidylserine and phosphatidylmyoinositol were not separated from each other by this technique, but required further fractionation by the procedure of Skipski *et al.* (1962). Bisphosphatidylglycerol was contaminated with glycolipid and was purified on silica gel G by development with chloroform–methanol–water (65:25:1).

Preparation of Fatty Acid Methyl Esters. The purified phospholipids were treated with anhydrous 5% methanolic hydrogen chloride at 60° in sealed glass ampoules for 1.5 hr. Methylated derivatives were extracted by repeated washings with petroleum ether, bp 30–40°. The ethereal extract was reduced in volume and streaked onto a preparative thin layer plate for purification, using benzene development. Methyl esters were eluted with absolute methanol; and the residue, after evaporation of the solvent, was dissolved in a small volume of carbon disulfide for gas–liquid partition chromatography. Hydrogenation of the fatty acid methyl esters was carried out overnight in absolute ethanol with palladium oxide catalyst (Farquhar *et al.*, 1959).

Fatty acid methyl esters were saponified with 5% methanolic potassium hydroxide (O'Brien *et al.*, 1964). Purification by thin layer chromatography was required following this procedure.

Gas–Liquid Partition Chromatography. Fatty acid methyl esters were analyzed with an Aerograph Hy-Fi gas chromatograph, furnished with a hydrogen flame ionization detector. Stainless steel columns, 5 ft × 0.125 in. (outer diameter), were used. A polar column of 13% poly(diethyleneglycolsuccinate) on Anakrom ABS, operated at 182° with a nitrogen gas-flow rate of 22 cc/min, was employed; as well as a nonpolar column of 16.4% Apiezon L on Anakrom ABS, used at 230° and 25–35 cc of nitrogen/min. Peak areas were estimated from the height and width at one-half peak height or by a disk integrator. Quantitative results with standard methyl ester mixture H-104 agreed with the stated composition with a relative error of less than 2% for components constituting more than 5% of the mixture, and less than 10% for those in amounts less than 5% of the mixture.

Tentative assignments for each peak were obtained from a semilogarithmic plot of "relative retention times *vs.* chain length and degree of unsaturation" of standard mixtures of fatty acid methyl esters. Hydrogenation was used to check the chain length and unsaturation of each peak. Gas–liquid partition chromatography of the nonsaponifiable material in each methylated mixture was employed for detection of dimethylacetals.

Although methanolic hydrogen chloride can give spurious peaks on gas–liquid partition chromatography (Lindgren *et al.*, 1962), appropriate "blanks," with and without exposure to the silica gels and to Rhod-

TABLE I: Analysis of the Hydrophilic Constituents of the Deacylated Phospholipids.[a]

Component in Figure 2	Identity	Phosphorus	Total Glycerol	α-Linked Glycerol	Myoino-sitol	Choline	Nitrogen
1	Glycerylphosphoryl-choline	1.00	1.09			1.06	
2	Glycerylphosphoryl-inositol	1.00	1.04		1.14		
3	Glycerylphosphoryl-glycerol	1.00	2.1				0.13
4	Glycerylphosphoryl-ethanolamine	1.00	1.11				1.02
5	Glycerol 1,2-phosphate[b]	1.00	1.05				0.15
6	Inositol 1-phosphate[b]	1.00	0.04		0.93		
7	Glycerol 3-phosphate[b]	1.00	1.05	1.04			0
8	Glycerol 2-phosphate[b]	1.00	0.96	0.06			0
9	Bis(glycerylphosphoryl)-glycerol	1.00	1.53	1.13			
10	Glycerylphosphoryl[b] glycerolphosphate	1.00	1.05	0.59			
11	Glycerol phosphate	1.00	0.98	0.67			0.35
12	Glycerylphosphoryl-serine	1.00	1.06	0.87			0.95

[a] Values are given as molar ratio relative to phosphorus. [b] Artifacts produced by phosphodiester cleavage during deacylation.

amine 6G, gave no misleading results at the detector response attenuations used (×32 and ×64).

Results

Phospholipid Content of Pine Pollen. An average of 0.09% of the air-dried pollen was extracted as lipid phosphorus, which represents 0.12% of oven-dried pollen. Assuming an average molecular weight of 750, the total phospholipid content of the dry pollen was approximately 3% on a weight basis. Only 7% of the phospholipid fraction was nonsaponifiable. The neutral lipid fraction, which contained some glycolipids, was not investigated.

Characterization of the Deacylated Phospholipids. In Figure 1 is illustrated the preliminary column separation of the deacylated phospholipid mixture. Paper chromatography of peaks A–E gave the phosphorus-containing components 1–12 in Figure 2. With the exception of spots 5 and 8, these substances also reacted with the periodate–benzidine dip reagent. Peak A contained several fast- and slow-running components which reacted with periodate–benzidine, but not with the Hanes–Isherwood spray, and which probably represent glycolipid derivatives.

Most of the constituents in each peak could be separated from each other by preparative paper chromatography, using overnight development with the basic solvent mixture A. Spots 7 and 8 required development in this solvent system for 3 days, and components 11 and 12 were best separated in the acidic solvent mixture B over a 12-hr period. After the purification procedure with DEAE-Sephadex (see Experimental Procedure) each component was analyzed, the results of which are given in Table I.

Component 1 cochromatographed on paper with standard glycerylphosphorylcholine in solvents A and D, gave a positive reaction for quaternary ammonium ion, and on hydrolysis in 2 N hydrochloric acid at 120° for 48 hr yielded glycerol, inorganic phosphate, and choline. Components 2 and 3 were chromatographically identical with glycerylphosphorylmyoinositol and glycerylphosphorylglycerol, respectively, using solvent systems A and C, and on acid hydrolysis gave only the products expected for each of these two compounds. Component 4 was ninhydrin positive and ran with glycerylphosphorylethanolamine in solvents A and C. Its acid hydrolysate contained glycerol, inorganic phosphate, and ethanolamine.

Component 5 matched none of the common deacylated phospholipids. Its high R_F value suggested either glycerylphosphorylmethanol or glycerol 1,2-phosphate; and, when compared to synthetic standards of these two compounds, it cochromatographed with the latter in solvent system A and in 1-butanol–propionic acid–water (2:1:1.4) (Maruo and Benson, 1959). Consistent with this assignment, component 5 consumed a negligible amount of periodate under the experimental conditions described by Kabat and Mayer (1964).

Zone electrophoresis and paper chromatography,

along with the molar ratios presented in Table I, indicated that spot 6 is myoinositol 1-phosphate and spots 7 and 8 represent glycerol 3- and -2-phosphates, respectively. Since phosphatidic acid was not detected in the intact phosphatides, it is assumed that the glycerol phosphates are artifacts of the deacylation procedure, as are the myoinositol 1-phosphate and glycerol 1,2-phosphate.

The analysis of component 9 in Table I strongly suggested that it was the deacylated product of bisphosphatidylglycerol. Paper chromatography and electrophoresis of the intact and acid-hydrolyzed compound with appropriate standard verified this assignment. Isomerization of glycerylphosphorylglycerol in acid to produce bis(glycerylphosphoryl)glycerol is reported to occur in deacylated phospholipid mixtures (Brundish *et al.*, 1965). That component 9 is the product of such a reaction is not likely, particularly since bisphosphatidylglycerol itself was present in fresh extracts of pollen phospholipid.

Component 10 had a glycerol:phosphate ratio suggesting (glycerolphosphoryl)glycerol phosphate. A standard of this compound was prepared from deacylated bisphosphatidylglycerol, as described under Materials. The two compounds had identical chromatographic properties. Component 10 was degraded by the modified Barry procedure to a product which had a glycerol:phosphate ratio of 1:2. However, this material contained two components which chromatographed on paper with standard glycerol 1,2-diphosphate and glycerol 1,3-diphosphate. The 1,2 isomer represented approximately 21% of the mixture and the 1,3 isomer, 79%. It was concluded that component 10 was a mixture of the two isomers 1-(glycerol-3′-phosphoryl)-glycerol 2- and -3-phosphates, and is an artifact from the breakdown of bisphosphatidylglycerol rather than being the deacylation product of phosphatidylglycerol phosphate.

Compounds such as component 10 have been reported infrequently in deacylated phospholipid fractions (Brundish *et al.*, 1965), although they could be derived from phosphatidylglycerol phosphate. The latter is thought to be an intermediate in the synthesis of phosphatidylglycerol in liver mitochondria (Kiyasu *et al.*, 1963) while a nonsaponifiable phospholipid with two ether-linked hydrocarbon chains and a hydrophilic constituent of 1-(glycerol-3′-phosphoryl)-glycerol 3-phosphate has been found in *Halobacterium cutirubrum* (Kates, 1965).

Upon paper chromatography in solvent G, component 11 gave two phosphate-positive spots, migrating with glycerol 1- and -2-phosphates. Hydrolysis of the material in acid gave only glycerol and inorganic phosphate. Thus, component 11 had the properties of glycerol phosphate; and, on the basis of chromatographic data and its ratio of α-linked glycerol:phosphate seen in Table I, appeared to be a mixture of isomers.

Since glycerol 2- and -3-phosphates were eluted from DEAE-Sephadex in peak D (Figure 1), component 11 must represent a trace of glycerol phosphate produced by degradation of glycerylphosphorylserine after peak E was collected.

The cation present with glycerol phosphate is known to influence its partition properties during paper chromatography (van Heyningen and Pirie, 1958), which could explain the failure of component 11 to correspond exactly to the reference standards. Table II lists the migration properties of different salt forms of glycerol phosphate in solvents A and B. In contrast, the particular salt form of glycerol phosphate did not affect its elution from a DEAE-Sephadex (carbonate) column, in the presence or absence of added bis(glycerylphosphoryl)glycerol or glycerylphosphorylserine. Finally, component 12 had chromatographic properties identical with glycerylphosphorylserine in solvent systems A and B, gave a positive reaction with ninhydrin, and on acid hydrolysis yielded glycerol, inorganic phosphate, and serine.

TABLE II: Paper Chromatography of Glycerol Phosphate Salts.

Compound	Cation	Rate of Migration[b] Solvent A	Solvent B
Glycerol 3-phosphate	NH_4^+	1.00	1.00
	Na^+	0.80	
	K^+	0.60	
	CHA$^+$[a]	1.00	1.66
	Ca^{2+}	Streak	0.39
Glycerol 2-phosphate	NH_4^+	1.19	
	Na^+	0.82	
	CHA$^+$	1.19	

[a] CHA denotes cyclohexylamine. [b] Given relative to glycerol 3-phosphate (NH_4^+).

Thin Layer Chromatography of the Phosphatides. Thin layer chromatography of the phospholipid fraction, obtained by the silicic acid slurry technique, gave the pattern in Figure 3. Table III shows the composition of the mixture. Identification of each phospholipid was based on chromatographic comparison with standard phosphatides, and was confirmed by

TABLE III: Composition of Total Phospholipid Fraction.

Phosphatide	% of Total
Phosphatidylcholine	60
Phosphatidylethanolamine	18
Phosphatidylmyoinositol	13
Phosphatidylglycerol	6
Bisphosphatidylglycerol	2
Phosphatidylserine	1

TABLE IV: Fatty Acid Composition of Individual Phosphatides.

Phospholipid	Fatty Acids							% Unsaturated
	16:0[a]	18:0	18:1	18:2	18:3	20:2	Other	
Phosphatidylcholine	18[b]	2	17	52	4	2	6	79
Phosphatidylethanolamine	21	<1	8	50	8	4	8	78
Phosphatidylmyoinositol	40	<1	17	39	<1	<1	4	58
Phosphatidylglycerol	38	2	10	38	3	<1	9	54
Bisphosphatidylglycerol	22	5	14	29	2	5	22[c]	72
Phosphatidylserine	41	4	22	18	<1	8	8	53

[a] Number of carbon atoms to number of double bonds. [b] Per cent of total fatty acids in phosphatide. [c] The major component had a retention time of 5.4 relative to palmitic acid.

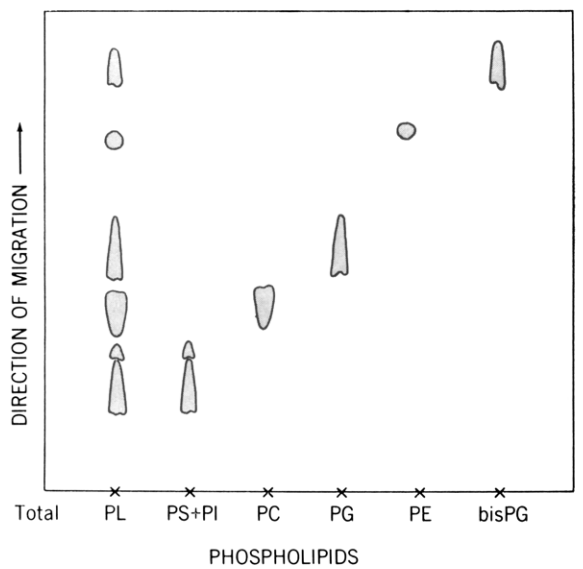

FIGURE 3: Thin layer chromatogram of the crude phosphatide mixture and of the purified components isolated from pine pollen, developed with chloroform–methanol–water (65:25:4), and sprayed with "molybdenum blue" reagent. PL represents the phospholipid mixture under study; PS, phosphatidylserine; PI, phosphatidylmyoinositol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; and bisPG, bisphosphatidylglycerol.

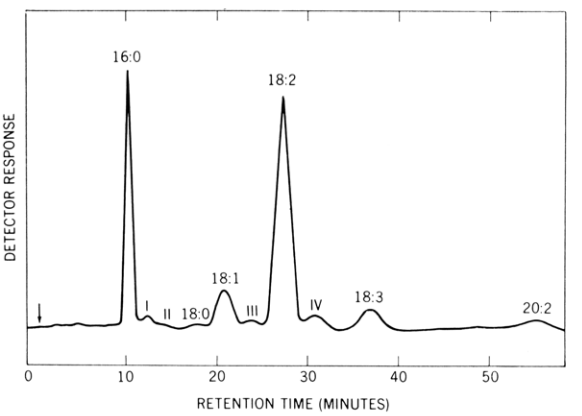

FIGURE 4: Gas chromatogram of the fatty acid methyl esters of phosphatidylethanolamine on 13% poly(diethyleneglycolsuccinate) at 182°, 22 ml/min of nitrogen carrier gas and detector response attenuation × 64. The arrow indicates the point of sample injection. Carbon number and number of double bonds is indicated above each identified peak. Peaks I–IV were not identified.

analysis of the deacylated lipid. No lyso derivative was observed in freshly prepared phospholipid extracts, although lysophosphatidylcholine was detected in stored phosphatide preparations.

Fatty Acid Composition. Table IV summarizes the fatty acid content of each phospholipid. Figure 4 is the elution pattern of the methyl esters obtained from phosphatidylethanolamine. The minor components which were not identified are labeled I–IV. Peak I had a retention time (1.27) relative to palmitic acid on the polar column suggestive of palmitoleic acid, but on Apiezon L it showed a relative retention time of 1.32, far greater than that of palmitoleic acid. The peak was not affected by hydrogenation, but disappeared upon saponification. The peaks labeled III and IV in Figure 4 disappeared after hydrogenation, as well as when the mixture was saponified with potassium hydroxide. The percentages of palmitic acid and arachidic acid, after hydrogenation, were within 1–2% of those found for the sums of the respective parent compounds in the original mixture. After hydrogenation, the stearic acid fraction was 7% higher than the combined C_{18} peaks that were identified before hydrogenation (Table IV). When peaks III and IV are added to the C_{18} fraction total, the value obtained is within 1% of the amount of stearic acid found in the hydrogenated mixture. On a plot of log of the relative retention times of each component on the polar column against the corresponding values on Apiezon

L (James, 1960), peaks III and IV fall into the C_{18} family of fatty acid methyl ester.

Hydroxy fatty acid methyl esters do not appear in elution patterns such as that seen in Figure 4, because these compounds would be removed during the purification procedure on thin layer chromatography (Morrison and Smith, 1964). When the area on the thin layer plate normally containing hydroxy fatty acid methyl esters was extracted and subjected to gas–liquid partition chromatography, no peak appeared within the C_{16}–C_{22} range. Scott and Strohl (1962) also note the absence of such acids in the lipids of pine pollen.

Fatty aldehyde dimethylacetals were not observed on thin layer chromatograms developed with benzene. When the total crude fatty acid methyl ester preparation was saponified and extracted with petroleum ether (bp 30–60°), no appreciable amount of dimethylacetal was detected by gas–liquid partition chromatography of the extract.

Discussion

Ponderosa pine pollen contains the phosphatidyl derivatives of choline, ethanolamine, glycerol, myoinositol, and serine, as well as bisphosphatidylglycerol. These phosphatides, and their fatty acids, are typical of those generally observed in higher plants (Allen and Good, 1965). The seed fats of the *Pinacae* belong to those of a group of plants which contain large amounts of oleic, linoleic, and/or linolenic acids, with smaller quantities of palmitic and stearic acids (Shorland, 1963). Although all of the phosphatides found in this study have been reported in higher plants, phosphatidylserine is found much less frequently than the others in the phanerogamous plants, possibly because it usually occurs in such small amount (Benson and Maruo, 1958; Kates, 1959).

The percentage of unsaturated fatty acid found in each phospholipid (Table III) is noteworthy. Approximately 75% of the fatty acids in phosphatidylcholine, phosphatidylethanolamine, and bisphosphatidylglycerol was unsaturated; while about 55% were unsaturated in phosphatidylmyoinositol, phosphatidylglycerol, and phosphatidylserine. Phosphatidylcholine and phosphatidylethanolamine are synthesized in many tissues by the reaction of diglyceride with CDP[1]-choline and CDP-ethanolamine, respectively (Kennedy, 1957). On the other hand, phosphatidylmyoinositol (Paulus and Kennedy, 1960), phosphatidylglycerol (Kiyasu *et al.*, 1963), and, in one instance, phosphatidylserine (Kanfer and Kennedy, 1964) are synthesized *via* a different pathway involving CDP-diglyceride. The biosynthesis of bisphosphatidylglycerol is not yet understood. It is possible that the differences in the two groups of phospholipids, distinguished by their content of unsaturated fatty acids, may reflect differences in the source of the diglyceride component found in them (Rhodes, 1964). Clearly, however, this correlation extends only roughly to a comparison of individual fatty acids, and the differences in saturation reflect mainly the balance between palmitic and linoleic acid.

Studies of the epidemiology (Cummings *et al.*, 1959) and experimental pathology (Lindner *et al.*, 1962) of sarcoidosis have suggested a possible relationship between pine pollen and the granulomatous disease. Since several branched-chain fatty acids are known to produce tubercles similar to those found in this disease (Lederer, 1961), the possible presence of such fatty acids was of interest in the present study. However, in neither this work nor that of Scott and Strohl (1962) has a branched-chain fatty acid been identified in pine pollen.[2]

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References

Abramson, D., and Blecher, M. (1965), *Biochim. Biophys. Acta 98*, 117.

Allen, C. F., and Good, P. (1965), *J. Am. Oil Chemists' Soc. 42*, 610.

Arndt, F. (1957), *in* Organic Syntheses, Coll. Vol. II, Blatt, A. H., Ed., New York, N. Y., Wiley, p 165.

Ballou, C. E., Vilkas, E., and Lederer, E. (1963), *J. Biol. Chem. 238*, 69.

Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.

Benson, A. A., and Maruo, B. (1958), *Biochim. Biophys. Acta 27*, 189.

Bouveng, H. O. (1963), *Phytochemistry 2*, 341.

Bouveng, H. O. (1965), *Acta Chem. Scand. 19*, 953.

Bregoff, H. M., Roberts, E., and Delwiche, C. C. (1953), *J. Biol. Chem. 205*, 565.

Brockeroff, H. (1963), *J. Lipid Res. 4*, 96.

Brown, D. M., Hall, G. E., and Higson, H. M. (1958), *J. Chem. Soc.*, 1360.

Brown, D. M., and Stewart, J. C. (1964), *J. Chem. Soc.*, 5362.

Brundish, D. E., Shaw, N., and Baddiley, J. (1965), *Biochem. J. 97*, 37C.

Crestfield, A. M., and Allen, F. W. (1955), *Anal. Chem. 27*, 422.

Cummings, M. M., Dunner, E., and Williams, J. H. (1959), *Ann. Internal Med. 50*, 879.

Dittmer, J. C., and Lester, R. L. (1964), *J. Lipid Res. 5*, 126.

Farquhar, J. W., Insull, W. W., Jr., Rosen, P., Stoffel, W., and Ahrens, E. H., Jr. (1959), *Nutr. Rev. 17*, 1.

Folch, J., Lees, M., Sloane-Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.

Gordon, H. T., Thornburg, W., and Werum, L. N. (1956), *Anal. Chem. 28*, 848.

Hanahan, D. J., and Olley, J. N. (1958), *J. Biol. Chem.*

[1] Abbreviation used: CDP, cytidine diphosphate.

[2] One of the referees suggests that component I in Figure 4 might be a branched C_{17} fatty acid.

231, 813.
Hanes, C. S., and Isherwood, F. A. (1949), *Nature 164*, 1107.
James, A. T. (1960), *Methods Biochem. Analy. 8*, 1.
Kabat, E. A., and Mayer, M. M. (1964), Experimental Immunochemistry, 2nd ed, Springfield, Ill., Thomas, p 547.
Kanfer, J., and Kennedy, E. P. (1964), *J. Biol. Chem. 239*, 1720.
Kates, M. (1959), *Biochem. Biophys. Res. Commun. 1*, 238.
Kates, M., Yengoyan, L. S., and Sastry, P. S. (1965), *Biochim. Biophys. Acta 98*, 252.
Kennedy, E. P. (1957), *Ann. Rev. Biochem. 26*, 119.
Kiyasu, J. Y., Pieringer, R. A., Paulus, H., and Kennedy, E. P. (1963), *J. Biol. Chem. 238*, 2293.
LeCocq, J., and Ballou, C. E. (1964), *Biochemistry 3*, 976.
Lederer, E. (1961), *Pure Appl. Chem. 2*, 587.
Lindgren, F. T., Nichols, A. V., Freeman, N. K., and Willis, R. D. (1962), *J. Lipid Res. 3*, 390.
Lindner, A., Kutkam, T., Sokatch, J. R., and Hammarsten, J. F. (1962), *Exptl. Mol. Pathol. 5*, 470.
Long, C., and Staples, D. A. (1961), *Biochem. J. 78*, 179.
Maruo, B., and Benson, A. A. (1959), *J. Biol. Chem. 234*, 254.
Morrison, W. R., and Smith, L. M. (1964), *J. Lipid Res. 5*, 600.
Nilsson, M. (1956), *Acta Chem. Scand. 10*, 413.
Nilsson, M., Ryhage, R., and von Sydow, E. (1957), *Acta Chem. Scand. 11*, 634.
O'Brien, J. S., Fillerup, D. L., and Mead, J. F. (1964), *J. Lipid Res. 5*, 329.
Paulus, H., and Kennedy, E. P. (1960), *J. Biol. Chem. 235*, 1303.
Rhodes, D. N. (1964), *in* Metabolism and Physiological Significance of Lipids, Dawson, R. M. C., and Rhodes D. N., Ed., New York, N. Y., Wiley, p 621.
Scott, R. W., and Strohl, M. J. (1962), *Phytochemistry 1*, 189.
Shaw, G., and Yeadon, A. (1966), *J. Chem. Soc. 1C*, 16.
Shorland, F. B. (1963), *in* Chemical Plant Taxonomy, Swain, T., Ed., New York, N. Y., Academic, p 253.
Skipski, V. P., Peterson, R. P., and Barclay, M. (1962), *J. Lipid Res. 3*, 467.
Snell, E. E. (1950), *in* Vitamin Methods, Vol. I, Gyorgy, P., Ed., New York, N. Y., Academic, p 445.
Stanley, R. G., and Loewus, F. A. (1964), *in* Pollen Physiology and Fertilization, Linskens, H. F., Ed., Amsterdam, Elsevier, p 128.
Stanley, R. G., and Young, L. C. T. (1962), *Nature 196*, 1228.
Strohl, M. J., and Seibel, M. K. (1965), *Phytochemistry 4*, 383.
Tano, S., and Takahashi, H. (1964), *J. Biochem. (Tokyo) 56*, 578.
Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950), *Nature 166*, 444.
van Heyningen, R., and Pirie, A. (1958), *Biochem. J. 68*, 18.
Wagner, H., Hörhamer, L., and Wolff, P. (1961), *Biochem. Z. 334*, 175.
Wheeldon, L. W., and Collins, F. P. (1958), *Biochem. J. 70*, 43.