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Characterization of Inositol-containing Phosphosphingolipids from Tobacco Leaves

ISOLATION AND IDENTIFICATION OF TWO NOVEL, MAJOR LIPIDS: N-ACETYLGLUCOSAMIDOGLUCURONIDOINOSITOL PHOSPHORYLCERAMIDE AND GLUCOSAMIDOGLUCURONIDOINOSITOL PHOSPHORYLCERAMIDE[1]

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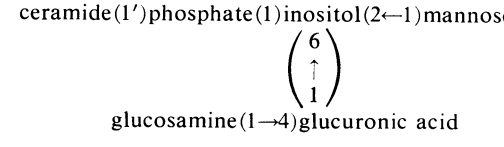
ABSTRACT

A method for a large scale extraction of phosphoglycosphingolipids from the leaves of *Nicotiana tabacum* L. has been developed. The phosphosphingolipid concentrate consists of a dozen or more polar lipids as judged by thin layer chromatography. Two of these lipids were purified by chromatography on porous silica beads and partially characterized. These lipids are formulated as: N-acetylglucosamidoglucuronidoinositol phosphorylceramide and glucosamidoglucuronidoinositol phosphorylceramide. Although not fully characterized, the other lipids in the concentrate are inositol-containing phosphosphingolipids with a higher carbohydrate content.

Glycosphingolipids are thought to play a significant role in cell surface function in animals. Considerable information is available concerning their chemistry, biosynthesis, and degradation (38). The neutral glycosphingolipids are present in most animal cells and are composed of uncharged heterooligosaccharides of various chain lengths glycosidically linked to ceramide (N-fattyacylsphingosine). Ceramide-monohexosides (cerebrosides) have been characterized from higher plants, animals and fungi (38). Neutral ceramide-oligosaccharides, to our knowledge, have not been reported in higher plants, although a ceramide-tetrasaccharide has been isolated from the fungus, *Neurospora crassa* (19). Gangliosides, negatively charged glycosphingolipids, which derive their charge from one or more sialic groups glycosidically linked to the oligosaccharide portions of various ceramide-oligosaccharides (38), are widespread in animal cells but have not been reported to occur in plants.

A negatively charged glycosphingolipid that does occur in plants is phytoglycolipid, so named by Carter and co-workers (6) who have carried out pioneering investigations in this area. They showed that phytoglycolipid occurred in seeds from corn, flax, and soybeans (6), as well as in bean leaves (11). Phytoglycolipid is apparently unique to plants, although related substances have been reported in fungi (19, 29).

Alkaline hydrolysis of corn phytoglycolipid gave rise to a mixture of oligosaccharides and phosphooligosaccharides (5), as well as ceramide and ceramide-phosphate (8). The ceramide moieties of phytoglycolipid were composed of saturated hydroxy-fatty acids, mainly C-24, attached to either 4-hydroxysphinganine (phytosphingosine) or 4-hydroxy-8-sphingenine (dehydrophytosphingosine) (11). Studies showing the predominant oligosaccharide from the corn phytoglycolipid hydrolysate to be 2-mannosido-6-[D-glucosamido-(1-4)-D-glucuronido]inositol as well as earlier work led Carter *et al.* (12) to propose the following structure for corn phytoglycolipid:



Phytoglycolipid preparations heretofore obtained are apparently mixtures, since alkaline hydrolysis yields mixtures of longer and shorter oligosaccharides (5). However, this point will be best resolved when pure components are available. Nothing is known about the metabolism or function of these lipids.

In order to study further the chemistry, metabolism, and biological function of these unique plant phosphosphingolipids, convenient methods need to be developed for their extraction, purification, and analysis. In this paper, we report a convenient method for the extraction and concentration of phosphosphingolipids from tobacco leaves. A thin layer chromatographic procedure we have developed shows this group to be quite heterogeneous. Two of the major phosphosphingolipids, PSL-I and PSL-II, obtained in pure form have been partially characterized as novel lipids.

MATERIALS AND METHODS

Harvesting and Homogenization of Leaves. Mature field-grown leaves of *Nicotiana tabacum* L. var Ky. 14 were harvested early in the morning towards the end of the growing season (August 17–29). The leaves were packed in crushed ice immediately after harvesting and were kept at 0 to 5 C through the homogenization step which was carried out within 3 to 6 hours after harvest.

Three kg of leaves, chopped into roughly 1 to 2 inch^2 pieces, 195 ml of 100% (w/v) trichloroacetic acid and 605 ml of 95% ethanol were homogenized in a 1-gallon Waring Blendor for 1 min. After standing for 30 min, the pH of the homogenate

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was brought to 6.5 to 7.5 with 78 ml of concentrated NH_4OH. The neutralized homogenate was stored at −20 C until used for the next step. Each liter of homogenate corresponded to 0.8 kg of fresh leaves.

Sphingolipid Extraction from Leaf Homogenate. The procedure for the preparation of sphingolipid concentrate from the leaf homogenate is outlined in Figure 1. Twenty-liter batches of homogenate were processed as follows. To each liter of leaf homogenate was added 649 ml of ethanol, 268 ml of diethyl ether, 53.6 ml of pyridine, and 5 ml of concentrated NH_4OH to bring the pH up to 8.5. This mixture was refluxed at 60 C for 45 to 60 min with continuous stirring. The extract was filtered through four layers of cheesecloth while still warm. The filtrate was rapidly cooled by pumping through an ice-cooled stainless steel coil, and the pH was adjusted to about 5 by adding glacial acetic acid. The precipitate was most conveniently sedimented by storing the mixture at 5 C for 7 to 10 days.

Most of the supernatant could be siphoned off and was discarded, leaving a mixture consisting of 10% precipitate by volume. This was slurried and mixed with one-tenth volume of Hyflo Super Cel (Johns-Manville Co.) and one-fourth volume of acetone. The mixture was filtered by suction. The resulting filter cake was resuspended in another one-fourth volume of acetone and refiltered. This process was repeated twice more to get rid of most of the pigments. The acetone-washed precipitate (stage III, Fig. 1) was air dried, slurried up in a small volume of solvent A ($CHCl_3$:CH_3OH:H_2O, 16:16:5, v/v), poured into a glass chromatography column, and eluted with solvent A containing 0.05 M CH_3COONH_4 until sphingolipids no longer appeared as judged by TLC. About 25 liters of this eluate results from processing 80 kg of leaves. To the eluate one-half volume of CH_3OH was added, and the resulting precipitate was allowed to sediment for 2 days at 5 C. The supernatant was removed by siphoning and was kept at −20 C for 2 more days to obtain more precipitate. The combined 5 C and −20 C sedimented precipitate slurry was centrifuged to remove the supernatant remaining after siphoning. The precipitate obtained was air dried and stored in a vacuum dessicator at 5 C (stage V).

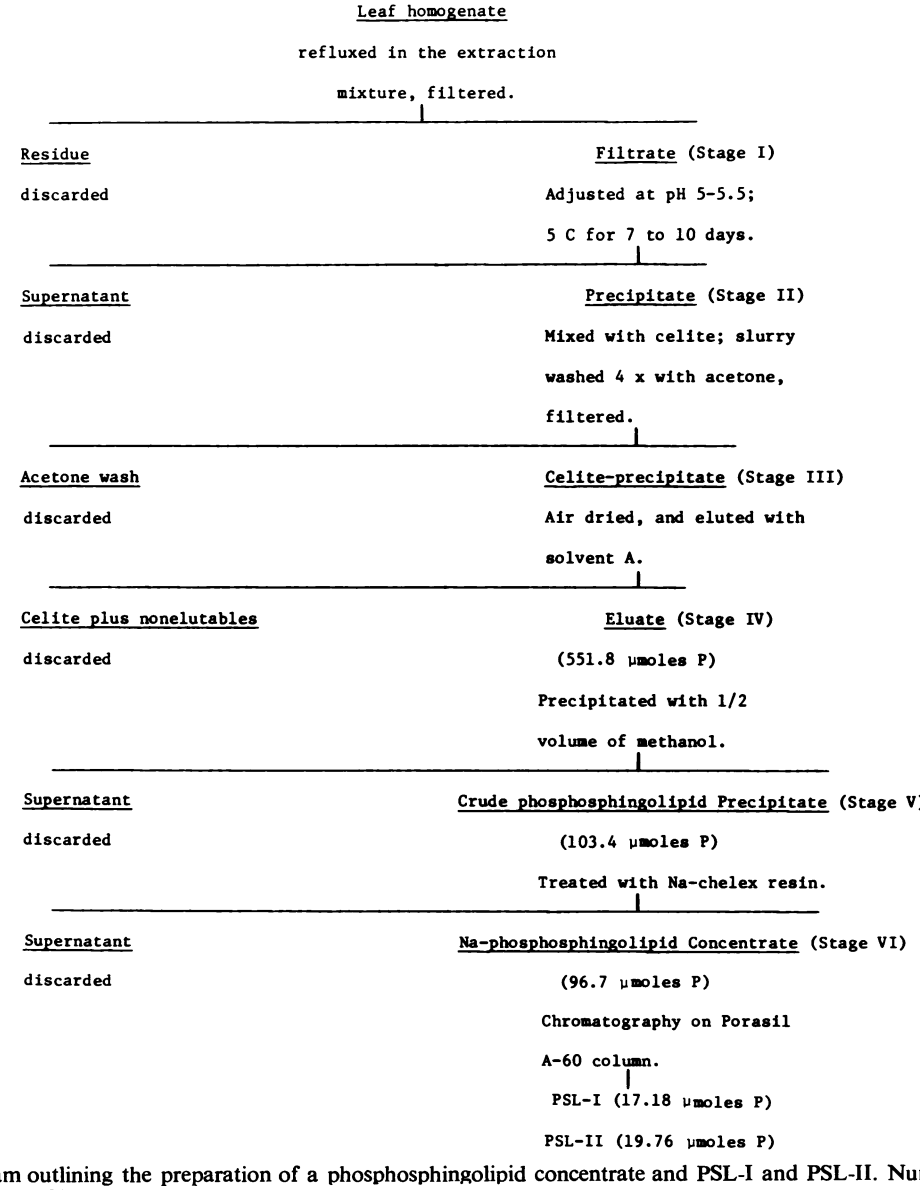

FIG. 1. Flow diagram outlining the preparation of a phosphosphingolipid concentrate and PSL-I and PSL-II. Numbers in parentheses are yield of P/kg fresh weight of leaves.

Preparation of Na-phosphosphingolipid Concentrate. In a typical experiment, stage V precipitate (1.06 mmole of P) was suspended in 100 ml of pyridine-H_2O (3:7, v/v) and passed through a 40-ml column of Chelex 100 resin (Na^+, 100–200 mesh, Bio-Rad Laboratories) equilibrated with the same solvent adjusted to pH 8.8. The column was further eluted with 70 ml of this solvent, and to the eluate was added two volumes of acetone. After standing overnight at 5 C, the precipitate, obtained by centrifugation, was washed twice with acetone and air dried (stage VI) yielding 0.99 mmol of P.

Column Chromatography of Phosphosphingolipid Concentrate. Porous silica beads (Porasil A 60, 75–125 μm, Waters Associates, Inc.) treated with base, as previously described (29), were used throughout for analytical and preparative column chromatography. Analytical chromatography was carried out by loading an aqueous suspension of the Na-phosphosphingolipid concentrate (1.3 ml, 40 μmoles of P) on a 1.14 × 300 cm Porasil column equilibrated with $CHCl_3$. The sample was followed with 15 ml of methanol. The column was then eluted with 4355 ml of a linear gradient of $CHCl_3$:CH_3OH:H_2O. The first gradient chamber contained 2600 ml of $CHCl_3$:CH_3OH:H_2O (59:37.5:3.5, v/v) and the second gradient chamber contained 2741 ml of $CHCl_3$:CH_3OH:H_2O (46:42:12, v/v). The column was further eluted with solvent A. The flow rate was 4.3 ml/min, and the volume of each fraction was 67 ml. The temperature of the column was maintained at 43 C. Aliquots from fractions were taken for TLC and for P determination. The P profile from such a column is shown in Figure 2, left. Fractions were pooled as indicated.

Preparative chromatography of the P-sphingolipid concentrate to obtain PSL-I and PSL-II was carried out with more heavily loaded columns eluted in a stepwise fashion. Seventy-five ml of the Na-P-sphingolipid concentrate dissolved in tetrahydrofuran-H_2O (3:1, v/v), 456 μmoles of P, was loaded on a 2.54 × 216 cm column equilibrated with $CHCl_3$. The column was maintained at 43 C and was eluted with $CHCl_3$:CH_3OH:H_2O (v/v/v) as follows: fractions 1 to 5, 65:40:0; 6 to 10, 65:40:3; 11 to 30, 56.4:38.4:5.8; 31 to 72, 54.5:39.4:7.3; and 73 to 92, 43:43:14. The volume of each fraction was 190 ml, and the flow rate was 18.5 ml/min. The P profile is shown in Figure 2, right.

Preparation of Acetate Form of DE52-cellulose. Sixty g of DE52-cellulose, microgranular preswollen (Whatman) were soaked twice in distilled H_2O, and the fines were removed. The DE52-cellulose was treated with 300 ml of 5% NaOH for 30 min, washed with distilled H_2O until the wash was neutral, treated with 300 ml of 10% H_3CCOOH for 30 min, and was again washed with distilled H_2O until the wash was neutral. The above was rinsed twice with 300 ml of CH_3OH, once with 300 ml of solvent A, and was then stored in solvent A.

Concentration of Porasil Column Fractions. The pooled fractions from the Porasil columns were passed through columns of acetate form DE52-cellulose (7.5 μmoles P/ml DE52-cellulose). All the sphingolipids were retained. PSL-I, PSL-II, and fraction A were eluted with one column volume of 1 M CH_3COONH_4 in solvent A, and fractions B and C were eluted with one column volume of 2 M CH_3COONH_4 in solvent A. The sphingolipid(s) were precipitated by adding one-half volume of CH_3OH to the eluate. After storage overnight at 5 C, the precipitate was separated by centrifugation and was washed with acetone. The washed precipitate was redissolved in solvent A and was reprecipitated by adding one-half volume of CH_3OH. This process was repeated twice to remove all CH_3COONH_4. The final products were air dried and were stored in a vacuum dessicator at 5 C.

Paper, Thin Layer, and Gas Chromatography. Chromatography of sugars was done on 589 Orange Ribbon paper (Carl Schleicher and Schuell, Inc.) in l-butanol-pyridine-H_2O (6:4:3, v/v) (16). The spots were located with a *p*-anisidine-phthalic acid spray (24). Phosphooligosaccharides were chromatographed on paper or cellulose thin layer in l-butanol-pyridine-NH_4OH-H_2O (6:4:4:1, v/v), and the spots were located with ninhydrin spray.

Lipids were chromatographed on silica gel thin layers (Quantum Industries PLQ, 1000 μm and LQ, 250 μm) in $CHCl_3$:CH_3OH:4 N NH_4OH (9:7:2, v/v) without or with 0.2 M CH_3COONH_4. The chromatograms were sprayed with Rhodamine to locate the lipid spots. Glycolipid spots were visualized by spraying the rhodamine-sprayed chromatograms with orcinol reagent (28). Long chain bases were chromatographed on silica gel thin layer in $CHCl_3$:CH_3OH:2 N NH_4OH (40:10:1 v/v) (23) and located with ninhydrin spray. Chromatography of fatty acid esters was done on silica gel thin layer in benzene-$CHCl_3$-glacial CH_3COOH (90:10:1), and the spots were visualized by rhodamine spray.

Gas chromaography was done on a Packard Series 7500 chromatograph fitted with a hydrogen flame ionization detector. Argon was carrier gas at 30 ml/min for acetic acid, 60 ml/min for long chain alcohols, long chain bases, sugars, and inositol, and at 110 ml/min for fatty acid esters. A column (182.9 cm × 4 mm) of OV-1 on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories) was used at 230 C for silylated long chain bases and sugars, at 175 C for silylated long chain alcohols, and at 205 C for silylated inositol. The same size

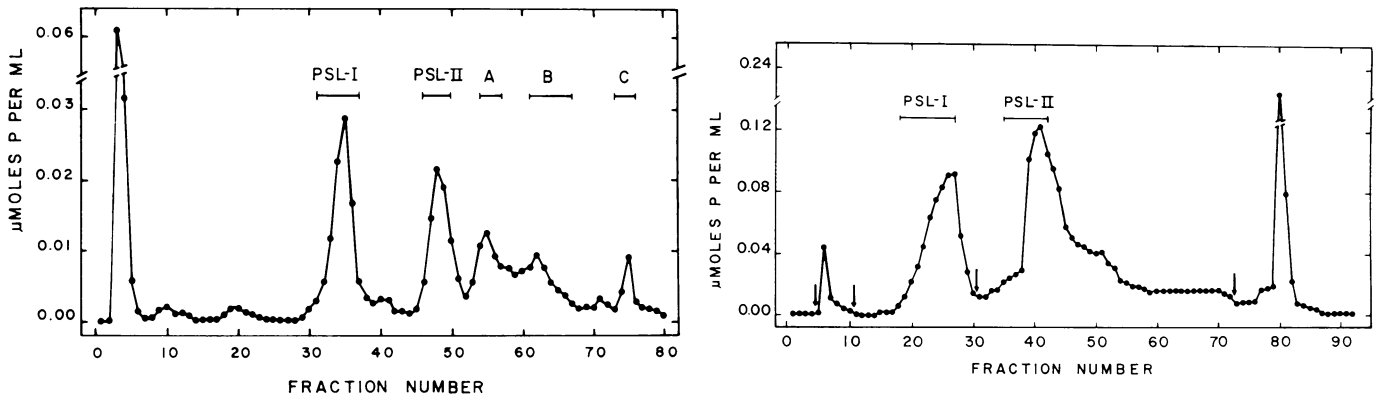

FIG. 2. Porasil column chromatography of P-sphingolipid concentrate. Left: chromatographic results obtained with lightly loaded column eluted with gradient as described under "Materials and Methods"; right: chromatographic results obtained with heavily loaded preparative column eluted in a stepwise fashion as described under "Materials and Methods." The arrows indicate solvent changes.

column packed with OV-17 on 60–80 mesh Gas-Chrom Q (Applied Science Laboratories) at 230 C was used for the estimation of silylated hydroxy-fatty acid methyl esters. Acetic acid estimation was done using the same size column packed with 80–100 mesh Porapak Q (Waters Associates, Inc.) coated with 2% (w/w) of 85% H_3PO_4 at 205 C (21). The acetic acid-containing sample was prepared by treatment of 0.8 μmole of PSL-I and PSL-II with 0.5 ml of 1 N HCl for 1.5 hr at 100 C. After centrifugation, a 2-μl aliquot was injected.

Quantitative Assays for Inositol. Inositol was estimated gas chromatographically after strong acid hydrolysis according to Wells *et al.* (40) as modified by Smith and Lester (29). Inositol was also estimated enzymatically using inositol dehydrogenase (Sigma Chemical Co.) by a slight modification of the method described by Weissbach (39). The assay mixture consisted of 0.3 ml of 1 M Na_2CO_3 buffer (pH 9.5), 0.2 ml of 0.1 M NAD, and 0.5 ml of substrate (0.05–0.15 μmole of inositol or unknown sample). Increase in absorbance at 340 nm was determined after incubation at 30 C for 30 min.

Other Analytical Procedures. Carbon, hydrogen, and nitrogen analyses were done by Galbraith Laboratories, Inc., Knoxville, Tenn. Phosphorus was determined after perchloric acid digestion according to Bartlett (2). Pi was measured without digestion. Sodium was measured by flame photometry after perchloric acid digestion. Carbohydrates were measured by phenolsulfuric acid method (15) with galactose as standard. Sialic acid was measured by the method of Svennerholm (32). Hexuronic acid determination was done by the phenylphenol method of Blumenkrantz and Asbose-Hansen (4) and by the carbazole method of Bitter and Muir (3). Rondle and Morgan's method (22) was employed for the estimation of hexosamine. Quantitative estimation of long chain base was done by a modification (29) of the method described by Siakatos *et al.* (27). Fatty acids were measured as their methyl esters by the method of Dittmer and Wells (14) with the 2-hydroxy-C_{24} acid as standard.

Acid Hydrolysis of PSL-I and PSL-II. For fatty acid and long chain base analysis, the lipids were hydrolyzed in concentrated HCl:CH_3OH:H_2O (3:29:4, v/v) at 78 C for 18 hr (9). Free fatty acids and their methyl esters were extracted in diethyl ether. The remaining hydrolysate was made alkaline with concentrated NH_4OH, dried, and taken up in a small volume of aqueous 0.05 N KOH solution. Long chain bases were extracted after adding isopropanol and benzene to get a final composition benzene-isopropanol-water (72:19.8:8.2, v/v).

To ensure the methylation of fatty acid, the fatty acid extract was dried, taken up in 14% methanolic BF_3, and heated in a steam bath for 3 min followed by extraction with petroleum ether. The fatty acyl methyl ester and long chain base fractions were silylated (7) and analyzed by gas chromatography along with appropriate standards.

Isolation of Phosphooligosaccharides after Basic Hydrolysis of PSL-I and PSL-II. Lipid samples were hydrolyzed in aqueous 0.5 N KOH at 100 C for 6 hr. The hydrolysate was neutralized with 5 N CH_3COOH and was extracted in a mixture having the composition, water-methanol-chloroform-toluene (2:1.5:2:0.5). The aqueous phase, which contained about half the original P was dried, redissolved in H_2O, and was passed through a small column of Dowex-50 (H^+) resin packed in H_2O. The P-oligosaccharide fraction was eluted with H_2O, dried *in vacuo,* redissolved in a small volume of H_2O, and adjusted to about pH 8 with 4 N NH_4OH. It was purified on a 0.6 × 81 cm AG1-X2 (HCO_3^-) resin (200–400 mesh, Bio-Rad Laboratories), pre-equilibrated with 60 ml of 0.1 M NH_4HCO_3, pH 7.9, followed by 30 ml of H_2O. The column was eluted with the following schedule: fractions 1 to 35, 0.2 M NH_4HCO_3; fractions 36 to 55, 0.3 M NH_4HCO_3; fractions 56 to 70, 0.5 M NH_4HCO_3. All fractions (approximately 9 ml) were assayed for total P. The fractions in the P peak were pooled and desalted by treatment with Dowex 50 (H^+) resin and then dried *in vacuo.*

Identification of Sugars. For hexosamine identification, lipid samples (about 1 μmole) were hydrolyzed with 0.5 ml of 5 N HCl for 3 hr at 100 C. The hydrolysate was diluted to 5 ml with H_2O and extracted twice with 3 ml of diethyl ether and then twice with 3 ml of petroleum ether. The aqueous fraction was neutralized by passing through 1 × 4 cm column of AG 1-X4 (CO_3^{2-}) followed with 12 ml of H_2O. Glycine was added as a reference standard prior to analysis on a Technicon Model NC-1 amino acid Autoanalyzer.

Identification of sugars in sphingolipids, as well as in derivatives in which the carboxyl of hexuronic acid was reduced, was carried out by the procedure of Desnick *et al.* (13) involving methanolysis of the lipids and subsequent gas chromatography of the trimethylsilylethers of the methylglycosides.

Nitrous Acid Degradation of Lipids and Phosphooligosaccharides. The procedure employed was adapted from the work of Shively and Conrad (26). Samples (0.02 μmole) as well as standards of glucosamine and N-acetylglucosamine were taken up in 10 μl of 0.1 N H_2SO_4 to which were added 5 μl of 5.5 M $NaNO_2$. After 10 min at room temperature, 10 μl of 1 M Na_2CO_3 were added, followed by 50 μl of 481 mM [^{3}H] $NaBH_4$ (81 mCi/mmole, New England Nuclear Corp.). After incubation at 50 C for 40 min, the excess borohydride was destroyed with 100 μl of 0.75 N H_2SO_4. A reagent blank was run in parallel.

Aliquots of the reaction mixtures (10 μl) were spotted as a 1.27 cm streak on Whatman No. 1 paper and developed in a descending fashion with ethyl acetate:HCOOH:CH_3-COOH:H_2O (18:1:3:4, v/v) for 18 hr. ^{3}H was detected by scintillation spectrometry after each lane was cut into 2.54 × 1.27 cm pieces and added to a Triton-toluene solution (33). Glucitol was run as a marker and was detected by periodate treatment of the chromatogram (37).

Preparation of Carboxyl-reduced Lipids. The acid form of PSL-I and PSL-II was prepared by passing a solution of the Na-lipid through a small column of Dowex 50 (H^+) resin. The reduction procedure was adapted from Taylor *et al.* (34). To 5 μmoles of lipid dissolved in 2 ml of H_2O was added 1 ml of aqueous 0.3 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. The solution was stirred at room temperature for 2 hr and then 5 μl of octyl alcohol followed by 226.8 mg of solid $NaBH_4$ were added. After stirring at 50 C for 6 hr, 9.6 ml of CH_3OH and 9.6 ml of $CHCl_3$ were added, giving the final composition, $CHCl_3$:CH_3OH:H_2O (16:16:5, v/v). This solution was neutralized with 20 ml of packed Dowex 50 (H^+) resin (Bio-Rad Laboratories). The resin slurry was filtered, and the eluate was dried under vacuum. The residue was redissolved in 25 ml of solvent A and passed through a small column of acetate form DE52-cellulose. The carboxyl-reduced lipid was eluted from the DE52-cellulose column with 0.5 M CH_3-$COONH_4$ in solvent A. The eluate was mixed with Dowex 50 (H^+) resin to remove CH_3COONH_4, filtered, and dried *in vacuo.* Reduction of the hexuronic acid was more than 90% complete as judged by the hexuronic acid assay on reduced and unreduced lipid.

Hydrolysis of Carboxyl-reduced Lipid and Chromatography of Products. Samples of carboxyl-reduced lipid (about 1 μmole) were hydrolyzed with 0.5 ml of 2 N HCl at 100 C for 3 hr. To the hydrolysate, 0.5 ml of H_2O was added and it was extracted

twice with 2 ml of diethyl ether. The aqueous phase was evaporated to dryness, taken up in 1 ml of H_2O, and was chromatographed with a mixture of [^{14}C]inositol-1- and 2-phosphates as an internal standard on a AG 1-X2 ($HCOO^-$, 200–400 mesh, Bio-Rad Laboratories) column eluted with a formate-borate gradient (20) at a flow rate of 1.2 ml/min. Aliquots from fractions 21 to 34 were taken for determination of radioactivity by scintillation counting (33). Phosphorus estimation was done on all fractions. [^{14}C]Inositol-1- and 2-phosphates were obtained by the action of an intestinal P-diesterase (1) on ^{14}C-labeled inositol phosphatidylinositol obtained from yeast grown on [UL-^{14}C]myoinositol (30). The cyclic 1,2-inositolphosphate thus formed was converted on long standing into the two monophosphate isomers (17).

Periodate Oxidation of Intact Lipids. A slight modification of Carter and Hirschberg's method (9) was employed to form long chain aldehydes by periodate oxidation of intact lipids and to reduce these aldehydes to their corresponding alcohols for gas chromatographic analysis. A sample of lipid (about 0.5 μmole) and a similar amount of hydroxysphinganine were dissolved in 0.35 ml of solvent A to which was added 0.35 ml of 0.057 M $NaIO_4$ in solvent A. After incubation in dark, at room temperature for 90 min, 1 ml of CH_3OH:H_2O (1:1, v/v) and 1.3 ml of $CHCl_3$ were added to the reaction mixture, and the contents were mixed and centrifuged. The $CHCl_3$ phase was dried, redissolved in 0.5 ml of CH_3OH, and treated with 0.5 ml of a $NaBH_4$ solution (1 g of $NaBH_4$ in 10 ml of 0.04 N NaOH) and it was allowed to stand at room temperature for 30 min. The reaction mixture was partitioned after adding 0.03 ml of 1 N HCl, 0.52 ml of H_2O, 0.3 ml of CH_3OH, and 1.6 ml of $CHCl_3$. The $CHCl_3$ phase was evaporated to dryness, 1 ml of H_2O was added, followed by two extractions with diethyl ether. The ether extract was evaporated to dryness, silylated (7), and analyzed by gas chromatography.

Periodate-Permanganate Oxidation of Intact Lipids. The double bond position in the long chain bases of PSL-I and PSL-II was established by identification of the fatty acids formed after a permanganate-periodate (36) oxidation of these lipids by the modified procedure of Youngs and Subbaram (41). To samples of lipids and standard hydroxysphinganine (about 1 μmole each) were added 0.4 ml of H_2O, 1.5 ml of *tert*-butanol, 0.1 ml of K_2CO_3 (8 mg/ml). To this solution was added 0.5 ml of the oxidizing mixture: 0.21 g of $NaIO_4$, 0.25 ml of 0.1 M $KMnO_4$, and H_2O to 10 ml. After incubating at 65 C for 2.5 hr in the dark 0.05 ml of ethylene glycol was added to decompose the reagents. After standing overnight at room temperature, the solution was evaporated to approximately 0.5 ml, to remove *tert*-butanol. The pH was brought to about 1 and contents were extracted three times with 3 ml of petroleum ether. The petroleum ether extract was evaporated to dryness, methylated with methanolic-BF_3, and analyzed by gas chromatography along with appropriate standards.

Reduction of Lipids. PSL-I and PSL-II were reduced with H_2 gas in presence of platinum oxide. About 5 μmoles of lipid were dissolved in 2 ml of tetrahydrofuran-H_2O (4:1, v/v), 3 mg of solid platinum oxide were added and gassed with H_2 for 2 hr at 35 C. Platinum oxide was removed by centrifugation and washed three times with 1 ml of 80% tetrahydrofuran.

Efficiency of Lipid Extraction Procedure. The efficiency of the sphingolipid extraction procedure used in the present study was assessed by measurement of hydroxysphinganine content in the extract and in the leaf homogenate residue after extraction. Samples of extract and residue were dried and were hydrolyzed by the method of Carter and Hirschberg (9). Long chain bases were extracted as described above and the extracts added to a Unisil (100–200 mesh, Clarkson Chemical Company Inc.) column, equilibrated with $CHCl_3$ and eluted in CH_3OH:$CHCl_3$:CH_3COOH (7:3:0.1, v/v). Dinitrophenyl derivatives of long chain bases were prepared by the method of Karlsson (18). The dinitrophenyl derivatives of unknown long chain bases along with dinitrophenyl derivative of hydroxysphinganine were chromatographed on silica gel impregnated paper (Whatman SG-81) in $CHCl_3$:CH_3OH (99:1, v/v). The yellow spots with the R_F of dinitrophenylhydroxysphinganine were cut out, eluted in CH_3OH, and the absorbance of the solution at 350 nm was determined.

RESULTS

Extraction of Sphingolipids and Preparation of Phosphosphingolipid Concentrate. No qualitative differences were observed between the sphingolipid mixture extracted from fresh tobacco leaves and that extracted from frozen homogenate of trichloroacetic acid-treated leaves, as judged by silica gel TLC of the lipid precipitate (stage II, Fig. 1) from the two kinds of extract. As a matter of convenience, trichloroacetic acid-treated frozen material was used for the large scale extraction in the present study wherein 550 kg of leaves were processed. As judged from the amount of hydroxysphinganine left in the residue after extraction, at least 80 to 90% of the total sphingolipids were extracted by the procedure described under "Materials and Methods." From the initial extract to the final P-sphingolipid concentrate (Fig. 1, stage VI), the purification steps were designed for convenience and monitored for a high yield of the desired P-sphingolipids by silica gel TLC. This procedure was derived from earlier work on the preparation of P-sphingolipids from *Saccharomyces cerevisiae* (29) and *Neurospora crassa* (19). A yield of about 100 μmoles of P/kg fresh weight was obtained at stage VI; a very rough estimate is that this represents about a 50 $\pm$ 20% yield. Stage VI lipid was mostly P-sphingolipid in nature since the ratio of long chain base to P was found to be 0.89.

Thin Layer Chromatography of Phosphosphingolipid Concentrate. Since no TLC systems for polar P-sphingolipids from plants have been described, a suitable system for tobacco P-sphingolipilds had to be developed. PSL-I, PSL-II, and other less polar lipids could be resolved on silica gel thin layer by $CHCl_3$:CH_3OH:4 N NH_4OH (9:7:2, v/v), a system previously described for sphingolipids from yeast (30). However, the sphingolipids comprising fractions A, B, and C (Fig. 2, left) streaked in this system, and the presence of ammonium acetate in the solvent system was necessary for the resolution of these lipids. Best results were obtained with 0.2 M ammonium acetate. Best resolution occurred when no more than 0.01 μmole of P-sphingolipid concentrate was spotted on a 1000 μm silica gel thin layer.

The final concentrate showed 15 rhodamine-positive spots and 10 orcinol-positive spots on silica gel thin layer (Fig. 3). Twelve of these spots were resistant to mild alkaline hydrolysis (Fig. 3), a characteristic of nonacylester-containing lipids, such as sphingolipids.

Column Chomatography of Phosphosphingolipid Concentrate. Column chromatography on base-treated porous silica beads had been successful in the isolation of fungal P-sphingolipids (19, 29) and was therefore applied to the purification of the tobacco P-sphingolipids. When an aqueous suspension of the P-sphingolipid concentrate was added to a Porasil column and eluted with a gradient of $CHCl_3$:CH_3OH:H_2O, the results shown in Figure 2 (left) were obtained.

Thin layer chromatography of the eluate fractions showed that the order of elution from the column was much the same as on the thin layer plates (Fig. 3). The major peaks marked PSL-I and PSL-II appeared to be homogeneous, whereas the subsequent fractions were heterogeneous. The chemical char-

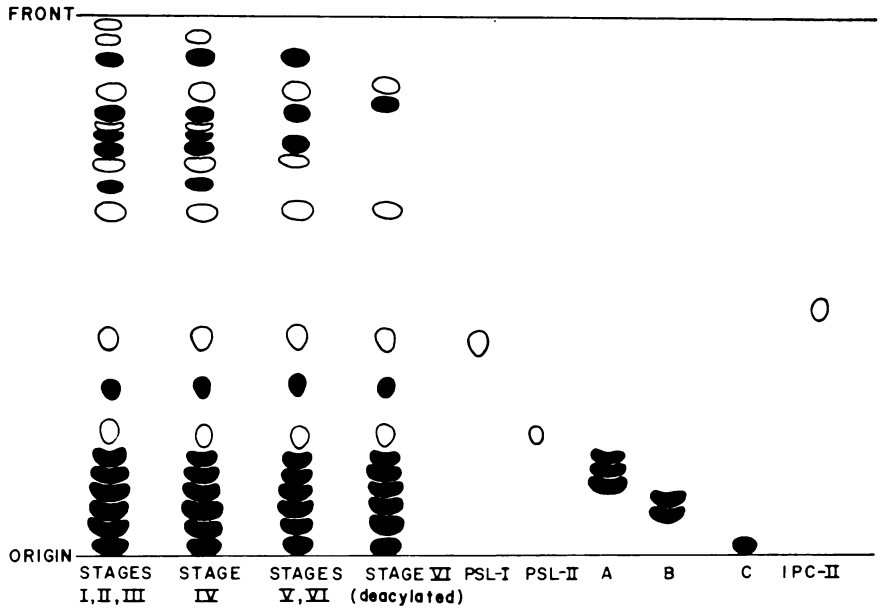

FIG. 3. TLC of fractions obtained from purification of P-sphingolipids. Purified intact lipids as well as crude fractions were chromatographed in $CHCl_3$:CH_3OH:4 N NH_4OH (9:7:2, v/v) containing 0.2 M ammonium acetate and were located by rhodamine spray (all indicated spots) as well as by orcinol reagent (filled spots). A, B, C were prepared as shown in Fig. 2 (left). IPC-II is an inositol phosphorylceramide (29).

Table I. *Composition of Phosphosphingolipid Fractions Isolated by Porasil Column Chromatography*

Sample	Long Chain Base	Inositol[1]	Carbohydrate[2]
	moles/mole P		
PSL-I	1.07	0.97	0.76
PSL-II	1.08	1.07	0.78
Fraction A	1.01	1.15	8.03
Fraction B	0.99	0.81	11.41
Fraction C	0.99	1.14	20.83

[1] Assayed with GLC.

[2] Assayed by phenol-H_2SO_4 method with galactose as standard.

acteristics of PSL-I and PSL-II, and fractions A, B, C (Fig. 2, left) are given in Table I. It can be readily seen that all are inositol-containing P-sphingolipids with equivalent amounts of P, long chain base, and inositol. The fractions most tightly bound to the Porasil are found to have increasingly high carbohydrate to P ratios (Table I). Since PSL-I and PSL-II could be readily purified and appeared to be novel components, we decided to obtain sufficient quantities of these lipids for further characterization. Chromatographic purification of PSL-I and PSL-II was achieved with more heavily loaded Porasil columns eluted with a stepwise elution schedule with the results shown in Figure 2 (right).

When the sample was applied as an aqueous suspension some of the PSL-I came out near the front (fractions 3–5) along with the less polar lipids (Fig. 2, left). However, when the sample was applied in tetrahydrofuran-H_2O (3:1), the first peak consisted of only the relatively nonpolar lipids and no PSL-I. The final products gave a single rhodamine-positive ninhydrin negative spot on two-dimensional silica gel TLC developed with the alkaline solvents (no ammonium acetate) as in Figure 3, followed with an acidic solvent: $CHCl_3$:CH_3OH:CH_3COOH:H_2O (15:6:4:1.6, v/v).

The P-sphingolipid concentrate as well as the purified P-sphingolipids were insoluble in conventional lipid solvents such as chloroform and could be dissolved only in solvents containing H_2O. The best solvent for the P-sphingolipd concentrate was found to be tetrahydrofuran-water (3:1, v/v); a solution containing 10 to 12 μmoles P/ml could be prepared. PSL-I was readily soluble in solvent A up to a concentration of 3 μmoles/ml. PSL-II, however, was only very sparingly soluble in solvent A but, dissolved up to about 3 μmoles/ml in pyridine-H_2O (3:7, v/v). Both PSL-I and PSL-II were soluble in H_2O (≥4 μmoles/ml) presumably as micelles. The more polar sphingolipids comprising fractions A, B, and C were very readily soluble in H_2O and could be dissolved up to at least a concentration of 15 μmoles P/ml.

Composition of PSL-I and PSL-II. As already indicated (Table I), the P-sphingolipid nature of PSL-I and PSL-II is evident from the presence of equimolar P and long chain base. The additional presence of one equivalent of fatty acid (Table II) suggests the presence of a ceramide moiety. Further evidence for this is that infrared (KBr) absorption bands observed for PSL-I (1630, 1530 cm^{-1}) and PSL-II (1620, 1525 cm^{-1}) are indicative of secondary amides. No ester absorption bands were evident, a fact consistent with the resistance of both PSL-I and PSL-II to mild alkaline methanolysis. Two colorimetric procedures show that both PSL-I and PSL-II contain an equivalent of uronic acid. Colorimetric hexosamine determination performed on HCl hydrolysates of PSL-I and PSL-II shows the presence of 1 mole of hexosamine. When the hydrolysates were analyzed by an amino acid analyzer, the only ninhydrin-positive peak observed was at the elution volume

Table II. *Quantitative Analysis of PSL-I and PSL-II*

Assay	PSL-I	PSL-II
	moles/mole P	
Hexuronic acid (carbazole)	1.08	0.97
Hexuronic acid (phenylphenol)	0.99	0.99
Hexosamine (2 N HCl, 1 hr, 100 C)	0.89	0.00
Hexosamine (6 N HCl, 18 hrs, 100 C)	1.04	0.92
Acetyl	1.04	0.015
Sodium	1.80	0.89
Fatty acid	0.99	1.03

expected for 2-amino-2-deoxy-D-glucose; in this system galactosamine and mannosamine are resolved from glucosamine. It was noted that hexosamine was more easily liberated from PSL-I than from PSL-II (Table II). This fact led us to suspect the presence of an N-acetylamino sugar in PSL-I and its absence in PSL-II, since it is well known that unacetylated hexosamines are more resistant to acid hydrolysis. Direct analysis for the presence of acetic acid in the HCl hydrolysates of these lipids showed PSL-I to have one acetyl group, whereas it is absent from PSL-II (Table II). Although direct proof is not available, we conclude that the glucosamine of PSL-I is N-acetylated. The results of Na analysis showing PSL-I with two Na and PSL-II with one Na are also consistent with this view, since an N-acetyl group would result in one less positive charge. Therefore, from the data thus far presented, these two lipids would appear to differ only by an acetyl group and can be formulated as:

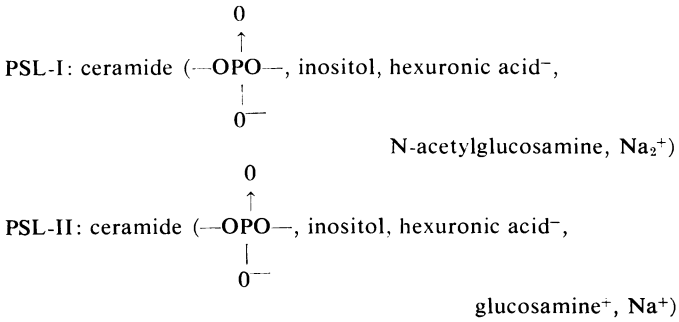

Table III. *Fatty Acid Composition of PSL-I and PSL-II*

Fatty Acid Methyl Ester	Retention Time			% Composition	
	Expected	Found		PSL-I	PSL-II
		PSL-I	PSL-II		
	min			*% detector response*	
OH:22	7.4	7.4	7.4	9.0	17.5
OH:23	10.1[1]	10.0	10.1	8.0	10.8
OH:24	13.8	13.8	13.8	55.4	52.1
OH:25	18.7[1]	18.7	18.8	17.9	12.6
OH:26	25.8	25.8	25.8	9.7	7.0

[1] Calculated from retention times of C-16, 18, 22, 24, 26 hydroxy-fatty acid methyl esters.

The presence of 0.8 carbohydrate equivalents in both PSL-I and PSL-II (Table I) is consistent with the above formulations since the phenol-H_2SO_4 procedure gives no response with hexosamine and in our hands glucuronic acid or galacturonic acid gives a color yield equivalent to 0.8 of that of galactose. Neither lipid contained sialic acid. No neutral hexoses or pentoses could be detected by the procedure of Desnick *et al.* (13).

Fatty Acid Composition of PSL-I and PSL-II. Silica gel TLC of the fatty acid methyl ester fraction from PSL-I and PSL-II showed only one rhodamine-positive spot at the R_F of monohydroxy-fatty acid methyl esters. Five hydroxy-fatty acids (C_{22}, C_{23}, C_{24}, C_{25}, and C_{26}) were identified by gas chromatography (Table III) with the retention times of the trimethylsilyl derivatives of the 2-hydroxy-methyl ester reference standards. In both PSL-I and PSL-II, more than 50% of the total fatty acid content was comprised of 2-hydroxy-C_{24} acid (Table III). There was no change in the retention times of the fatty acids derived from H_2-reduced PSL-I and PSL-II, indicating that all the fatty acids present were saturated.

Long Chain Base Composition of PSL-I and PSL-II. The long chain base fraction obtained from both PSL-I and PSL-II by $CH_3OH:HCl:H_2O$ hydrolysis showed one ninhydrin-positive spot with the R_F of hydroxysphinganine on silica gel TLC (Table IV). Gas chromatography of the silylated long chain base fraction of PSL-I and PSL-II showed two roughly equal peaks in both instances. In both instances, one peak corresponded to authentic 4-hydroxysphinganine, while the other peak had a slightly shorter retention time (Table IV). However, the long chain base fractions from lipid samples, which had been reduced with H_2, showed only one peak, corresponding to hydroxysphinganine. These results indicated the presence of hydroxysphinganine and a hydroxysphingenine in both PSL-I and PSL-II.

The location of the double bond in the hydroxysphingenine was ascertained by a permanganate-periodate oxidation of PSL-I and PSL-II. High yields of decanoic acid and pentadecanoic acid were obtained, the former presumably from 4-hydroxy-8-sphingenine and the latter from 4-hydroxysphinganine. Similarly, periodate oxidation and subsequent reduction of reaction products by $NaBH_4$ resulted in a high yield of pentadecanol, as well as a product with a retention time reasonable for a pentadecenol for both PSL-I and PSL-II (Table IV).

All the above results are consistent with the notion that the long chain base components of both PSL-I and PSL-II are a mixture of 4-hydroxysphinganine and 4-hydroxy-8-sphin-

Table IV. *Qualitative Identification of Long Chain Bases of PSL-I and PSL-II*

Procedure	R_F Values or Retention Times		Identity of Underivatized Products
	Products	Reference standards	
Thin layer chromatography of long chain base fractions[1]	R_F 0.25	R_F 0.25	4-D-Hydroxysphinganine[2]
Gas-liquid chromatography of TMS[3] derivatives of long chain base fractions[1]	8.7 min	8.7 min	4-D-Hydroxysphinganine
	8.3 min	? min	4-D-Hydroxy-8-sphingenine?
Gas-liquid chromatography of TMS derivatives of long chain base fractions[1] obtained from lipids reduced with H_2-PtO	8.7 min	8.7 min	4-D-Hydroxysphinganine
Methylesters of acids after permanganate-periodate oxidation	1.1 min	1.1 min	Decanoic acid
	12.9 min	12.9 min	Pentadecanoic acid
TMS derivatives of long chain alcohols produced by periodate borohydride treatment	13.5 min	13.5 min	Pentadecanol
	12.2 min	? min	Pentadecenol?

[1] Prepared by HCl-CH_3OH-H_2O hydrolysis.
[2] 4-D-Hydroxy-8-sphingenine presumed to have same R_F.
[3] Trimethylsilyl.

Table V. *Analyses of HCl Hydrolysates of Carboxyl-reduced PSL-I and PSL-II*

Lipid	Initial Lipid P	Water-soluble Products				
		Before phosphatase			After phosphatase	
		Total P	Pi	Free inositol	Pi	Free inositol
	μmoles					
Carboxyl-reduced PSL-I	2.0	1.8	0.14	0	1.8	1.9
Carboxyl-reduced PSL-II	1.9	1.7	0.18	0.3	1.6	1.7

genine. Furthermore, the facile formation of C-15 fragments by periodate oxidation of the intact lipids shows that the C-3 and C-4 hydroxyl groups of the long chain bases are unsubstituted.

Nature of Hexuronic Acid in PSL-I and PSL-II. Analysis of acid hydrolysates of PSL-I and PSL-II by gas chromatography did not show the presence of a hexuronic acid. It is well known that saccharides containing uronic acids and amino sugars are difficult to cleanly hydrolyze without destruction of the uronic acid. We, therefore, reduced the carboxyl group of the lipid prior to hydrolysis which should then yield a new neutral sugar. When the carboxyl of the lipids was reduced by the method of Taylor *et al.* (34), glucose in good yield could be detected in acid hydrolysates of both lipids by GLC. This observation showed that the hexuronic acid present in both PSL-I and PSL-II is glucuronic acid.

Inositol Phosphates from Degradation of PSL-I and PSL-II. Since the carboxyl reduced lipids underwent acid hydrolysis to give glucose in good yield, we also examined the P-containing water-soluble products of acid hydrolysis. Acid hydrolysis of the carboxyl-reduced PSL-I and PSL-II resulted in about 90% of the total P becoming water-soluble (Table V), mostly as organic P. Treatment of this fraction with alkaline phosphatase yielded equimolar amounts of Pi and free inositol, showing that inositol phosphomonoesters were present in the hydrolysate. Anion exchange chromatography of the hydrolysate with radioactive inositol-1- and 2-phosphates gave identical results for both PSL-I and PSL-II; all the P was recovered and about half was associated with inositol-1-P, whereas the other half was comprised of roughly equal amounts of inositol-2-P and a third peak which probably was another inositol phosphate isomer (Fig. 4). These observations indicated that all the phosphate of both PSL-I and PSL-II is linked to inositol, probably at the one position.

Characterization of Phosphooligosaccharides from Alkaline Hydrolysis of PSL-I and PSL-II. After strong alkaline hydrolysis of PSL-I and PSL-II, about 50% of the total P became water-soluble. Anion exchange chromatography (Fig. 5) of this water-soluble fraction showed a single P-containing peak at the same elution volume for PSL-I and PSL-II. Identity of these P-oligosaccharides was also shown by cellulose TLC. The strong base treatment can be expected to hydrolyze the N-acetyl group of PSL-I. Both isolated products were found to contain equimolar amounts of P, inositol, hexuronic acid, and hexosamine (Table VI). These facts argue strongly that aside from the acetyl group in PSL-I, the basic structure of the polar components of PSL-I and PSL-II is identical. Thus, each of the known polar components of both PSL-I and PSL-II are associated in a P-oligosaccharide which is presumably attached to the ceramide.

Since all the P is linked to inositol, the sequence of the polar components of these lipids is either P-inositol-glucuronic acid-glucosamine or P-inositol-glucosamine-glucuronic acid. If the unsubstituted glucosamine is terminal, then nitrous acid treatment would be expected to result in deamination and cleavage to produce mainly a 2,5-anhydrohexose (26) which can in turn be measured after reduction with [^{3}H]-NaBH$_4$ (26). Following the procedure of Shively and Conrad (26), we treated both intact PSL-I and PSL-II as well as both P-oligosaccharide products of alkaline hydrolysis with nitrous acid followed by reduction with [^{3}H]-NaBH$_4$. These reaction mixtures were chromatographed along with reaction mixtures from parallel runs with 2-amino-2-deoxyglucose and 2-N-acetyl-amino-2-deoxyglucose. PSL-II and both the P-oligosaccharides

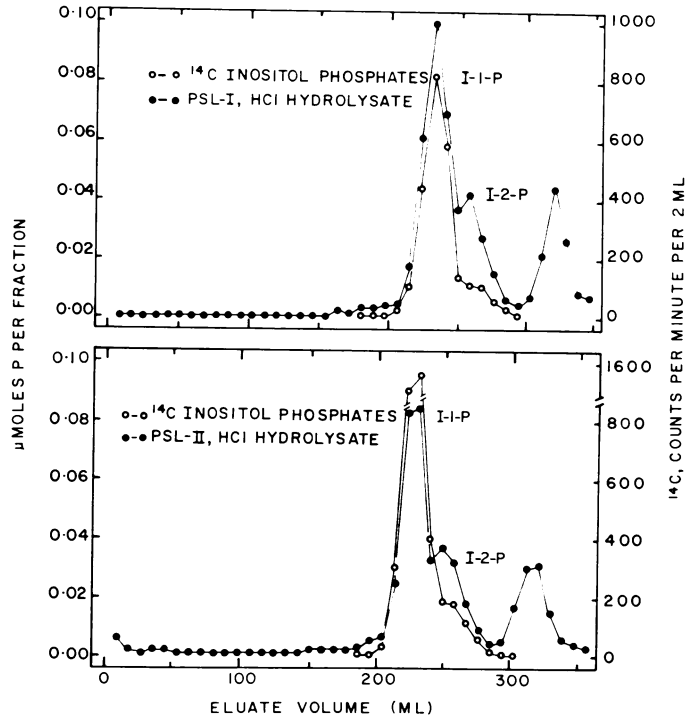

FIG. 4. Anion exchange chromatography of water-soluble products of HCl hydrolysis of carboxyl reduced PSL-I and PSL-II. Upper: PSL-I product was mixed with an authentic mixture of ^{14}C-inositol-1-P and inositol-2-P prior to chromatography as described under "Materials and Methods"; lower: chromatography as above of PSL-II product.

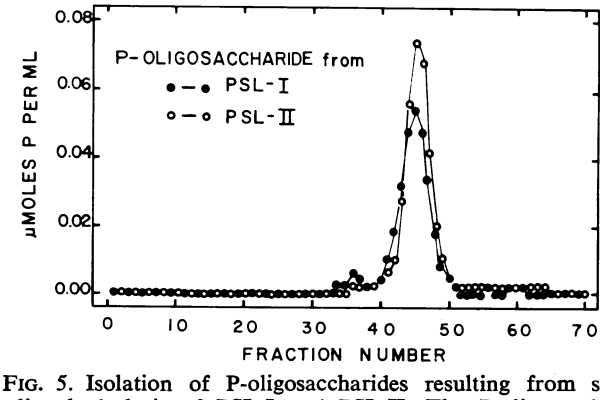

FIG. 5. Isolation of P-oligosaccharides resulting from strong alkaline hydrolysis of PSL-I and PSL-II. The P-oligosaccharide fractions were prepared and chromatographed on anion exchange columns eluted with ammonium bicarbonate as described under "Materials and Methods."

Table VI. *Chemical Composition of P-oligosaccharides Isolated from PSL-I and PSL-II after Strong Alkaline Hydrolysis*

Component	PSL-I (P-oligosaccharide)	PSL-II (P-oligosaccharide)
	moles/mole P	
Hexuronic acid[1]	1.18	1.21
Hexosamine	0.93	1.05
Inositol[2]	1.18	1.21

[1] Assayed by phenylphenol method (4).

[2] Assayed by enzymatic method after 41 hr hydrolysis with 6 N HCl at 105 C.

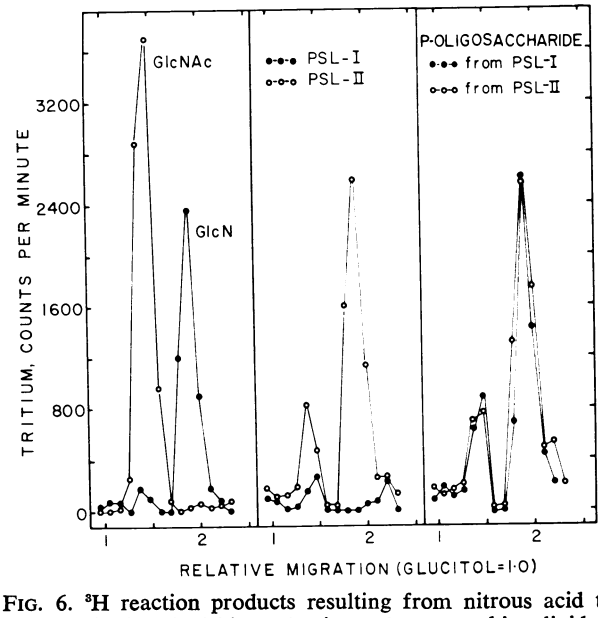

FIG. 6. ^3H reaction products resulting from nitrous acid treatment and ^3H-borohydride reduction of pure sphingolipids and P-oligosaccharides. Descending chromatography of the reaction mixtures was done on Whatman No. 1 paper in ethyl acetate: HCOOH:CH_3COOH:H_2O (18:1:3:4). The migration values have been expressed relative to that for glucitol on the same chromatogram. Notice the absence of the glucosamine product peak in PSL-I.

give the same products, with the same R_F as that obtained from glucosamine (Fig. 6), showing that glucosamine is terminal in each of these starting materials. The lack of this product from intact PSL-I is consistent with the presence of a N-acetylglucosamine moiety which would not be expected to undergo deamination. Thus the polar moiety of PSL-I appears to be -P-inositol-glucuronic acid-N-acetylglucosamine and the polar moiety of PSL-II is -P-inositol-glucuronic acid-glucosamine.

Elemental Analysis of PSL-I and PSL-II. Elemental analysis of purified preparations of PSL-I and PSL-II (Table VII) are in good agreement with the expected compositions, especially when the products are considered to contain a small percentage of H_2O.

DISCUSSION

The original method of Carter *et al.* (6) for the isolation of P-sphingolipids employed commercial lipid preparations which were subjected to alkaline hydrolysis to remove acylester lipids followed by solvent fractionation. Countercurrent distribution of commercial plant lipid preparations without resort to alkali treatment also resulted in purification of a P-sphingolipid fraction (10). More recently Carter and Koob (11) have extracted P-sphingolipids from bean leaves by refluxing in 70% ethanol (0.1 N in HCl) for 20 min; further purification was achieved by chromatography on DEAE-cellulose (11). It is not known whether this acidic extraction procedure results in some lipid breakdown, however, it seems prudent to employ an extraction method such as that herein described that avoids very harsh conditions. Our extraction employed appears to be efficient in that no more than 20% of the total hydroxysphinganine is left in the residue. This is only a rough estimate, since not all the phytosphingosine is associated with P-sphingolipid. It is also possible that the extracted P-sphingolipid does not have a representative distribution, the unextracted lipid being possibly quite polar. More direct methods are necessary to resolve this question.

Tobacco leaf P-spingolipids are present in amounts similar to other phospholipids (35). The amount of isolated P-sphingolipid concentrate (about 100 μmoles/kg fresh weight) is of comparable magnitude to the estimate of phytoglycolipid present in the crude extract from bean leaves (0.1% of dry weight) (11) given reasonable estimates of leaf moisture.

The proposed structures for the ceramide trisaccharides, PSL-I and PSL-II, as well as the properties of the partially purified tobacco P-sphingolipids indicate their close similarity to the phytoglycolipid studied by Carter *et al.* (6). Still to be determined is the precise linkage and stereochemistry of the oligosaccharide components of PSL-I and PSL-II. In fungi, related sphingolipids with the following compositions have been described: inositol P-ceramide; mannose-inositol P-ceramide; mannose-(inositol P)$_2$-ceramide; (inositol P)$_2$-ceramide (19, 29, 31).

As already indicated, individually purified components have not heretofore been obtained but based on the results of alkaline hydrolysis, Carter *et al.* (5) estimate that trisaccharides constitute approximately 9% of corn phytoglycolipid. Our work indicates that the ceramide trisaccharides, PSL-I plus PSL-II, constitute about 40% of the tobacco P-sphingolipids.

It is interesting that the presence of an acetyl group has such a great effect on the polarity of these compounds as indicated by their chromatographic behavior (Figs. 2 and 3) and their solubility properties. The diversity of the as yet unidentified tobacco P-sphingolipids could possibly be due to the presence of both acetylated and unacetylated variants of the

Table VII. *Elemental Composition of PSL-I and PSL-II*

	C	H	N	P	Na
	%				
PSL-I					
Found	52.84	8.64	1.90	2.24	3.07
Calculated[1], anhydrous	55.30	8.38	2.08	2.30	3.41
Calculated[1], ·3 H_2O	53.16	8.49	2.00	2.21	3.28
PSL-II					
Found	53.10	8.69	2.00	2.43	1.60
Calculated[2], anhydrous	56.10	8.87	2.18	2.41	1.79
Calculated[2], ·3 H_2O	53.84	8.96	2.09	2.31	1.72

[1] Calculated from the formula, $C_{62}H_{112}O_{24}N_2PNa_2$, based on a ceramide composed of a C-24 hydroxy fatty acid and equal amounts of 4-D-hydroxysphinganine and 4-D-hydroxy-8-sphingenine as well as one equivalent of inositol, N-acetylhexosamine, and hexuronic acid.

[2] Calculated from the formula $C_{60}H_{111}O_{23}N_2PNa$ and on components indicated above except that hexosamine replaces N-acetylhexosamine.

same lipid. It is not known whether any of the phytoglycolipid preparations of Carter *et al.* (6) contained N-acetyl groups. Amino sugars found in animal glycosphingolipids are invariably acetylated. Although the procedure used for isolation of PSL-I and PSL-II avoided harsh conditions, we cannot exclude the possibility that PSL-II was formed from PSL-I by postharvest deacylation. We plan to examine this possibility carefully.

Further work will be directed towards the isolation and further characterization of the more polar P-sphingolipids present in fractions A, B, and C (Fig. 3). Possibly similar substances have been observed by Carter and Kisic (10) who have presented evidence for the occurrence of glucosamine-free ceramide-P-polysaccharides in commercial preparations of seed lipids of corn, flax, soybean, and safflower. Analyses of these preparations whose purity was not established indicated a polysaccharide chain length of about 14 sugars. Chromatography on formaldehyde-treated paper was the only chromatographic method employed by Carter *et al.* (10, 11) which might have established purity of these P-sphingolipids; however, since this method apparently cannot distinguish between trisaccharide- and tetrasaccharide-containing lipids (10) it would be of doubtful value in assessing purity of even more complex lipids. The silica gel TLC procedure herein described may be helpful in monitoring the no doubt difficult purification of the very polar P-sphingolipids as well as possibly becoming the basis for a quantitative estimation.

We hope that the results and procedures described in this paper will be helpful in answering the many questions concerning the biological role of these unique and apparently ubiquitous plant lipids.

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LITERATURE CITED

1. Atherton, R. S., P. Kemp, and J. N. Hawthorne. 1966. Phosphoinositide inositolphosphohydrolase in guinea pig intestinal mucosa. Biochem. Biophys. Acta 125: 409–412.
2. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. J. Biol. Chem. 234: 466–468.
3. Bitter, T. and H. M. Muir. 1962. A modified uronic acid carbazole reaction. Anal. Biochem. 4: 330–334.
4. Blumenkrantz, N. and G. Asboe-Hansen. 1973. New method for quantitative determination of uronic acids. Anal. Biochem. 54: 484–489.
5. Carter, H. E., B. E. Betts, and D. R. Strobach. 1964. Biochemistry of the sphingolipids. XVII. The nature of the oligosaccharide component of phytoglycolipid. Biochemistry 3: 1103–1107.
6. Carter, H. E., W. D. Celmer, D. S. Galanos, R. H. Gigg, W. E. M. Lands, J. H. Law, K. L. Mueller, T. Nakayama, H. H. Tomizawa, and E. Weber. 1958. Biochemistry of the sphingolipides. X. Phytoglycolipide, a complex phytosphingosine-containing lipide from plant seeds. J. Amer. Oil Chem. Soc. 35: 335–343.
7. Carter, H. E. and R. C. Gaver. 1967. Improved reagent for trimethylsilylation of sphingolipid bases. J. Lipid Res. 8: 391–395.
8. Carter, H. E., R. H. Gigg, J. H. Law, T. Nakayama, and E. Weber. 1958. Biochemistry of the sphingolipides. XI. Structure of phytoglycolipide. J. Biol. Chem. 233: 1309–1314.
9. Carter, H. E. and C. B. Hirschberg. 1968. Phytosphingosines and branched sphingosines in kidney. Biochemistry 7: 2296–2300.
10. Carter, H. E. and A. Kisic. 1969. Countercurrent distribution of inositol lipids of plant seeds. J. Lipid Res. 10: 356–362.
11. Carter, H. E. and J. L. Koob. 1969. Sphingolipids in bean leaves (*Phaseolus vulgaris*). J. Lipid Res. 10: 363–369.
12. Carter, H. E., D. R. Strobach, and J. N. Hawthorne. 1969. Biochemistry of the sphingolipids. XVIII. Complete structure of tetrasaccharide phytoglycolipid. Biochemistry 8: 383–388.
13. Desnick, R. J., C. C. Sweeley, and W. Krivit. 1970. A method for the quantitative determination of neutral glycosphingolipids in urine sediment. J. Lipid Res. 11: 31–37.
14. Dittmer, J. C. and M. A. Wells. 1969. Quantitative and qualitative analysis of lipids and lipid components. Methods Enzymol. 14: 490–491.
15. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350–356.
16. French, D., D. W. Knapp, and J. H. Pazur. 1950. Studies on the sachardinger dextrins. VI. The molecular size and structure of the γ-dextrin. J. Amer. Chem. Soc. 72: 5150–5152.
17. Jungalwala, F. B., N. Freinkel, and R. M. C. Dawson. 1971. The metabolism of phosphatidylinositol in the thyroid gland of the pig. Biochem. J. 123: 19–33.
18. Karlsson, K. 1970. On the chemistry and occurrence of sphingolipid long-chain bases. *In:* C. C. Sweeley, ed., Chemistry and Metabolism of Sphingolipids. North-Holland Publishing Company, Amsterdam. pp. 6–43.
19. Lester, R. L., S. W. Smith, G. B. Wells, D. C. Rees, and W. W. Angus. 1974. The isolation and partial characterization of two novel sphingolipids from *Neurospora crassa:* (inositol-P)$_2$ ceramide and [(gal)sglc] ceramide. J. Biol. Chem. 249: 3388–3394.
20. Lester, R. L. and M. R. Steiner. 1968. The occurrence of diphosphoinositide and triphosphoinositide in *Saccharomyces cerevisiae*. J. Biol. Chem. 243: 4889–4893.
21. Mahadevan, V. and L. Stenroos. 1967. Quantitative analysis of volatile fatty acids in aqueous solution by gas chromatography. Anal. Chem. 39: 1652–1654.
22. Rondle, C. J. M. and W. T. J. Morgan. 1955. The determination of glucosamine and galactosamine. Biochem. J. 61: 586–589.
23. Sambasivarao, K. and R. H. McCluer. 1963. Thin layer chromatographic separation of sphingosine and related bases. J. Lipid Res. 4: 106–108.
24. Schweiger, A. 1962. Trennung einfacher Zucker auf Cellulose-schichten. J. Chromatog. 9: 374–376.
25. Shafizadeh, F. 1958. Formation and cleavage of the oxygen ring in sugars. Advan. Carbohyd. Chem. 13: 9–61.
26. Shively, E. and H. E. Conrad. 1970. Biochemistry of the nitrous acid deaminative cleavage of model amino sugar glycosides and glycosaminoglycuronans. Biochemistry 9: 33–43.
27. Siakatos, A. N., S. Kulkarni, and S. Passo. 1971. The quantitative analysis of sphingolipids by determination of long chain base as the trinitrobenzene sulfonic acid derivative. Lipids 6: 254–259.
28. Skipski, V. P. and M. Barclay. 1969. Thin layer chromatography of lipids. Methods Enzymol. 14: 545–546.
29. Smith, S. W. and R. L. Lester. 1974. Inositol-phosphoryl ceramide, a novel substance and the chief member of a major group of yeast sphingolipids containing a single inositol phosphate. J. Biol. Chem. 249: 3395–3405.
30. Steiner, S. and R. L. Lester. 1972. Studies on the diversity of inositol-containing yeast phospholipids: incorporation of 2-deoxyglucose into lipid. J. Bacteriol. 109: 81–88.
31. Steiner, S., S. Smith, C. J. Waechter, and R. L. Lester. 1969. Isolation and partial characterization of a major inositol-containing lipid in baker's yeast, mannosyl-diinositol, diphosphoryl-ceramide. Proc. Nat. Acad. Sci. U.S.A. 64: 1042–1048.
32. Svennerholm, L. 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. Biochim. Biophys. Acta 24: 604–611.
33. Talwalkar, R. T. and R. L. Lester. 1973. The response of diphosphoinositide and triphosphoinositide to perturbations of the adenylate energy charge in cells of *Saccharomyces cerevisiae*. Biochim. Biophys. Acta 306: 412–421.
34. Taylor, R. L., J. E. Shively, H. E. Conrad, and J. A. Cifonelli. 1973. Uronic acid composition of heparins and heparin sulfates. Biochemistry 12: 3633–3637.
35. Tso, T. C. 1972. Physiology and Biochemistry of Tobacco Plants. Dowden, Hutchinson, and Ross, Inc. Stoudsburg, Pa.
36. Von Rudloff, E. 1956. Periodate—permanganate oxidations. V. Oxidation of lipids in media containing organic solvents. Can. J. Chem. 34: 1413–1418.
37. Wawszkiewicz, E. J. 1961. A two-dimensional system of paper chromatography of some sugar phosphates. Anal. Chem. 33: 252–259.
38. Weigandt, H. 1971. Glycosphingolipids. Advan. Lipid Res. 9: 249–289.
39. Weissbach, A. 1958. The enzymatic determination of myo-inositol. Biochim. Biophys. Acta 27: 608–611.
40. Wells, W. W., T. A. Pittman, and H. J. Wells. 1965. Quantitative analysis of myoinositol in rat tissue by gas-liquid chromatography. Anal. Biochem. 10: 450–458.
41. Youngs, C. G. and M. R. Subbaram. 1964. Determination of the glyceride structure of fats: gas liquid chromatography of oxidized glycerides. J. Amer. Oil Chem. Soc. 41: 218–221.