Isolation of Six Novel Phosphoinositol-Containing Sphingolipids from Tobacco Leaves†

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ABSTRACT: A concentrate of phosphosphingolipids from tobacco leaves has been resolved into two groups by chromatography on diethylaminoethylcellulose. The first group eluted contains no acetyl residues, whereas the second group contains one *N*-acetyl/mol of phosphorus. Three lipids from each group have been purified by chromatography on Porasil columns. The chemical composition of these six novel lipids shows them to be related to the previously characterized phosphosphingolipids PSL-I, *N*-acetylglucosamine[glucuronic acid, inositol phosphate]ceramide and PSL-II, glucosamine[glucuronic acid, inositol phosphate]ceramide [Kaul, K., and Lester, R. L. (1975), *Plant Physiol. 55,* 120]. The novel lipids are: PSL-IA, PSL-I-[Ara_2Gal_2]; PSL-IB, PSL-I-[Ara_3Gal_2]; PSL-IC, PSL-I-[Ara_4Gal_2]; PSL-IIA, PSL-II-[Ara_3Gal]; PSL-IIB, PSL-II-[$Ara_{2or3}Gal_2$]; PSL-IIC, PSL-II-[Ara_2Gal_2Man]. The unusual finding of unacetylated hexosamine in the PSL-II group of lipids appears not to be an artifact, as suggested by experiments involving rapid harvesting and processing of the tobacco leaves. Some of the fractions obtained by chromatography on diethylaminoethylcellulose appear to contain phosphosphingolipids with 16 or more sugar residues.

The phosphosphingolipids of plants are a group of abundant glyco conjugates whose structure and function are not completely understood. We were able to prepare in high yield a concentrate of these lipids from leaves of *Nicotiana tabacum* (Kaul and Lester, 1975). The two most abundant components of this concentrate, differing only by an acetyl group, were purified and partially characterized: PSL-I, *N*-acetylglucosamine[glucuronic acid, inositol phosphate]ceramide, and PSL-II, glucosamine[glucuronic acid, inositol phosphate]-ceramide. Hsieh et al. (1978) have now more completely characterized PSL-I to be 2-deoxy-2-acetamido-D-glucopyranosyl(α1→4)-D-glucuronopyranosyl(α1→2)myoinositol-1-*O*-phosphoceramide.

We present evidence here that PSL-I and PSL-II are the simplest members of two families of related lipids, distinguished by whether the glucosamine component is acetylated. Taking advantage of the resulting charge difference, we have separated the two families by anion-exchange chromatography. We now report on the purification and characterization of six novel lipids, three from each family, containing various carbohydrate groups in addition to the PSL-I/II components.

Materials and Methods

For the purification of the phosphosphingolipids described in the present paper, the previously described sodium phosphosphingolipid concentrate (stage VI in Figure 1, Kaul and Lester, 1975) was the starting material.

Preparative Anion-Exchange Chromatography of the Phosphosphingolipid Concentrate. Acetate form DE-52 cellulose prepared as described earlier (Kaul and Lester, 1975) was poured in a 5 × 100 cm jacketed glass column, and the column was equilibrated with methanol-water (85:15). A suspension of the sodium phosphosphingolipid concentrate in pyridine-water (3:7) (4.77 mmol of P in 325 mL) was placed on the column at room temperature followed by 10 mL of methanol. The column was eluted at a rate of 9.9 mL/min, collecting 540-mL fractions with the following elution schedule. Fractions 1 and 2: methanol-water (85:15) at room temperature. From fractions 3 through 94, the column was eluted at 46 °C with an 80-h linear gradient from 0 to 0.03 M ammonium acetate in chloroform–methanol–water–acetic acid (16:16:5:0.37). The next four fractions (95–98) were eluted with a linear gradient from 0.03 to 0.2 M ammonium acetate in chloroform–methanol–water–acetic acid (16:16:5:0.37). The final solvent was pumped through the column up to fraction 102. The column was then eluted with about 1.5 column volumes of the same solvent containing 2.0 M ammonium acetate. Each fraction was assayed for total P (Figure 1).

Precipitation of Sphingolipids from the DEAE[1]-cellulose Column Fractions. All fractions were screened by chromatography on silica gel thin-layer plates (Quantum Industries PLQ, 1000 μm) in chloroform–methanol–4 N ammonium hydroxide (9:7:2) containing 0.2 M ammonium acetate. The spots were visualized by spraying with Rhodamine and orcinol reagents as previously described (Kaul and Lester, 1975). Based on the phosphosphingolipid composition of the column fractions, they were pooled into nine fractions, D_1–D_9, as indicated in Figure 1. Since the polar phosphosphingolipids were present only in fractions D_4–D_9, as shown by TLC (Figure 2), only these fractions were worked up. The phosphosphingolipids in fractions D_4 and D_5 were precipitated with 0.5 volume of acetone, and the phosphosphingolipids in fractions D_6–D_9 were precipitated with 1 volume of acetone. After 3 to 4 days at 4 °C to allow the precipitate to settle, the supernatant was siphoned off. As judged by the total P and carbohydrate content of the supernatant, 90% or more of the phosphosphingolipids were precipitated. The precipitate was washed three times with acetone, air dried, and stored at 4 °C in a desiccator.

Porasil Column Chromatography of DEAE-cellulose Column Fractions D_5 and D_7. Purification of some of the phosphosphingolipids of fractions D_5 and D_7 was done on columns of porous silica beads (Porasil A60, 37–75 or 75–125

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[1] Abbreviations used: PSL, phosphosphingolipid; TLC, thin-layer chromatography; DEAE, diethylaminoethyl.

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μm, Waters Associates, Inc.) treated with base, as previously described (Smith and Lester, 1974). Columns were eluted at room temperature with gradients of appropriate polarity constructed by mixing two solvents: chloroform–methanol–14 N ammonium hydroxide (387:387:27) (solvent I) and chloroform–methanol–14 N ammonium hydroxide–water (41.14:42.1:3.21:13.43) (solvent II). The solvent delivery system consisted of two M-6000A pumps controlled by a Model 660 programmer (Waters Associates) to yield the desired gradients. Decisions for pooling appropriate fractions were based on analysis of the eluates for pentose and total P and by TLC.

Continuous monitoring for pentose was done using a Technicon Autoanalyzer by a modification of the assay procedure described by Dische and Borenfreund (1957). Dische and Borenfreund's phloroglucinol reagent, without glucose (2.39 mL/min), column eluate (0.16 mL/min), and air (0.42 mL/min) were passed through a G2-type Technicon standard cactus fitting, mixed in a regular bore double mixing coil, passed through two 95 °C heating baths with double coils (each coil = 1.6 mm i.d. × 12 m), cooled in a jacketed cooling coil, and then passed through a Technicon C5 debubbler into the flow cell (0.92 mL/min) of a colorimeter with 550-nm filters. The absorbance at 550 nm was linear between 50 and 400 nmol/mL. Acid-flex tubing was used for the entry of the phloroglucinol reagent and the column eluate into the cactus fitting. Data from the pentose assay were plotted at the calculated fraction number (Figures 3 and 4). The appropriate column fractions were pooled and precipitated with acetone followed by standing at 4 °C. The precipitates were washed with acetone, air dried, and stored in a desiccator at 4 °C. A summary of the Porasil column chromatography of the various fractions employed to isolate the six sphingolipids described in this paper is given below. All samples were dissolved in pyridine–H_2O (85:15) prior to Porasil column chromatography. After application of the sample, solvent I was pumped through all columns for 2 min.

PSL-IA and IC. Fraction D_7 (0.73 mmol of P in 55 mL) was chromatographed on a 2.5 × 208 cm 75–125 μm Porasil column equilibrated with solvent I (column I, Figure 4). The column was eluted with 10.81 L of a linear gradient from 55% solvent I + 45% solvent II to 30% solvent I + 70% solvent II, 1.04 L of 30% solvent I + 70% solvent II, followed by 2.70 L of a linear gradient from 30% solvent I + 70% solvent II to 100% solvent II, and, finally, with 3.74 L of solvent II (Figure 4). Solvents were pumped at 9.9 mL/min. Based on silica gel TLC, the fractions containing pure PSL-IA and PSL-IC were pooled and processed as described above.

PSL-IB. Fractions from column 1 (above), rich in PSL-IB, were pooled, precipitated, and dried. A lipid sample containing 13 μmol of P in 2.2 mL was chromatographed on a 1.02 × 400 cm 37–75 μm Porasil column equilibrated with solvent I (column II). The column was eluted with 3.42 L of 35% solvent I + 65% solvent II, followed by 0.66 L of solvent II. Solvents were pumped at 12 mL/min, and 60-mL fractions were collected. Based on TLC, appropriate fractions containing PSL-IB were pooled and processed as described above.

PSL-IIB. Fraction D_5 (0.1 mmol of P in 10 mL) was chromatographed on a 1.1 × 200 cm 75–125-μm Porasil column equilibrated with solvent I (column III, Figure 3). The column was eluted at a rate of 9.9 mL/min with two linear gradients: 45% solvent I + 55% solvent II to 30% solvent I + 70% solvent II (1.18 L) followed by 0.2 L of 30% solvent I + 70% solvent II; then the second gradient, 30% solvent I + 70% solvent II to 100% solvent II (0.59 L) (Figure 3). The eluate was collected in 19.8-mL fractions. Fractions containing PSL-IIB were pooled and processed as described above.

PSL-IIA. Fractions from column III, which were rich in PSL-IIA, were pooled (Figure 3) and precipitated. The precipitate was dried, redissolved in pyridine–water (85:15), and chromatographed (17 μmol of P in 1.7 mL) on a 0.6 × 300 cm column of 37–75 μm Porasil equilibrated with solvent I (column IV). The column was eluted with 0.78 L of 35% solvent I + 65% solvent II. The solvents were pumped at a rate of 4 mL/min and 8-mL fractions were collected. Appropriate fractions were pooled after TLC.

PSL-IIC. Appropriate fractions from column III were pooled and precipitated. The precipitate was dried, taken up in pyridine–water (85:15), and chromatographed (11 μmol of P in 2 mL) on column V, a 1.02 × 400 cm 37–75-μm Porasil column equilibrated with solvent I. The column was eluted with 3.6 L of 30% solvent I + 70% solvent II followed by 0.72 L of solvent II. The solvent flow rate was 12 mL/min and 60-mL fractions were collected. Fractions containing PSL-IIC were pooled and processed as described above.

Gas Chromatographic Analysis of Sugars in Purified Lipids and Column Fractions. Sugars were analyzed as their alditol acetates by gas chromatography. Purified lipid samples (0.1–0.2 μmol of P) were hydrolyzed in 0.2 mL of 1 N H_2SO_4 at 100 °C for 12 h and further processed to obtain the alditol acetates as described by Laine et al. (1974).

The above samples were analyzed using a Perkin-Elmer 3920 gas chromatograph with a column (2 mm × 1 m) of 3% OV-275 on 100/120 mesh Chromosorb W AW (Supelco, Inc.) at 180 °C. Helium was the carrier gas at 50 mL/min. The hydrogen flame ionization detector response was integrated with a Perkin-Elmer M-2 calculating integrator. Mixtures of L-arabinose, D-galactose, and D-mannose in appropriate proportions were run in parallel for each lipid. D-Glucose was included as an internal standard.

Estimation of Mannose in PSL-IIC. Quantitative estimation of mannose was done by the method of Lee (1972). Approximately 0.07 μmol of lipid sample was hydrolyzed in a sealed glass tube in 0.2 mL of 1 N H_2SO_4 at 100 °C for 12 h. The hydrolysate was diluted with 0.2 mL of 0.15 M sodium borate buffer, pH 7.4. Half the sample was put on a Technicon Chromo-bead S (low-pressure type) column. The column was eluted with a linear gradient of sodium borate buffer, 0.15 (pH 7.4) to 0.4 M (pH 10.0). Sugars in the eluate were detected with orcinol–H_2SO_4 reagent. Rhamnose and xylose were used as internal standards.

Acetyl Analysis. A dry 0.1-μmol lipid sample was hydrolyzed with 0.06 mL of 1 N HCl in a screw-capped glass vial at 100 °C for 90 min. A 1 N HCl blank and a 0.1-μmol sample of *N*-acetylglucosamine were run in parallel. After hydrolysis, the samples were cooled to room temperature and a 2-μL aliquot was analyzed for acetic acid on a 1.83 m × 4 mm Carbopak B (Supelco, Inc.) column using a Packard series 7500 chromatograph fitted with a hydrogen flame ionization detector. The column temperature was 160 °C and the flow of argon through the column was 60 mL/min.

Other Analytical Procedures. Procedures followed for the colorimetric and gas chromatographic analysis of long-chain bases, fatty acids, and inositol and those for the colorimetric analysis of P, hexosamine, hexuronic acid, and total carbohydrate were the same as described earlier (Kaul and Lester, 1975).

Results

Anion-Exchange Chromatography of Phosphosphingolipid Concentrate. From our previous studies, it appeared possible that we were dealing with two families of phosphosphingolipids

Coupling of a Single Adenylate Cyclase to Two Receptors: Adenosine and Catecholamine†

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ABSTRACT: A detailed kinetic analysis on the rate of activation of adenylate cyclase by l-epinephrine and by adenosine, separately and combined, was performed. Both ligands were found to induce the activation of adenylate cyclase to its permanently active state in the presence of guanylyl imidodiphosphate (GppNHp). The activation followed strictly first-order kinetics. On the basis of these experiments, it was found that all of the enzyme pool can be activated by the β-adrenergic receptor, but only 60 to 70% of the enzyme can also be activated by an adenosine receptor. The remaining 30 to 40% cannot be activated by adenosine. While previous experiments have led us to conclude that the epinephrine receptor is uncoupled from the adenylate cyclase, it seems that the adenosine receptor is either precoupled to the enzyme or forms a long-lived intermediate of adenosine–receptor–enzyme complex. From the pattern of enzyme activation by the two ligands and GppNHp, it may be concluded that the two ligands, adenosine and the β-agonist, activate the adenylate cyclase through a common guanyl nucleotide regulatory site. This assertion is supported by the finding that both adenosine and l-epinephrine, in the presence of GTP, induce the reversal of the permanently active state, irrespective by which pathway the enzyme was activated.

Adenosine is a potent activator of adenylate cyclase in brain (Sattin and Rall, 1970), cultured human cell lines (Clark et al., 1974; Clark and Seney, 1976; Sturgill et al., 1975), and in neuroblastoma (Blume and Foster, 1975), as well as in rat brain (Gilman and Schrier, 1972), platelets (Mills and Smith, 1971), isolated bone cells (Peck et al., 1974), and in lymphocytes (Wolberg et al., 1975). Turkey erythrocyte adenylate cyclase was found by us also to be activated by adenosine (Sevilla et al., 1977). It is well established that turkey erythrocyte adenylate cyclase is activated by catecholamines through their interaction with β-adrenergic receptors. Thus, it became possible to study the question of activation of adenylate cyclase by two different receptors. This particular problem can be very well studied using the turkey erythrocyte adenylate cyclase system, as the latter is already well explored. Using the nonhydrolyzable GTP analogue, GppNhp,[1] one can study the process of enzyme activation as induced by either a β-agonist or by adenosine, separately, or by the two ligands combined. In this fashion, it becomes possible to analyze a number of basic questions: (a) Is the total pool of adenylate cyclase accesible to the two receptors? (b) Is the mode of enzyme activation by the two ligands additive in nature or do the receptors compete for the same pool of enzyme? (c) Is the pool of the regulatory guanyl nucleotide sites common to the two processes of activation?

Since a number of cell types possess adenylate cyclase and also respond to various hormones, these questions are of general significance.

Materials and Methods

Materials. Pyruvate kinase was from Sigma. PEP was the generous gift of Dr. Zvi Selinger, from our Department, and was neutralized by KOH to a pH of 7.4, and kept frozen at −20 °C.

Adenylate Cyclase Assay and Turkey Erythrocyte Membranes. Adenylate cyclase activity was measured at 37 °C according to Salomon et al. (1974). Assay was routinely performed for 20 min. The assay mixture contained 2 mM [α-^{32}P]ATP, 4 mM $MgCl_2$, 3 units of pyruvate kinase in saturating $(NH_4)_2SO_4$, 5 mM PEP, 10 mM KCl, and 40 mM Tris-HCl, pH 7.4.

Preparation of Membranes. Turkey erythrocyte membranes were prepared as previously described (Steer and Levitzki, 1975).

Activation of Adenylate Cyclase by l-Epinephrine and/or Adenosine and GppNHp. The time course of activation of the enzyme to its permanently active form was measured by preincubating separately 2.8 mg/mL membranes and a solution of 0.1 mM GppNHp and various concentrations of adenosine in 50 mM Tris-HCl buffer, pH 7.4, containing 2 mM $MgCl_2$ and 1 mM EDTA (TME buffer). After equilibration at 37 °C, the two solutions were mixed and, at various times, 70-μL portions were removed into 40 μL of an ice-cold solution containing 8 mM theophylline and 5×10^{-5} M *dl*-PPL. Theophylline is an adenosine antagonist (Clark and Seney, 1976) which stops the process of enzyme activation by adenosine and GppNHp. Theophylline, however, is unable to revert the enzyme from its permanently active state back to its inactive form. Thus, the relation between theophylline and adenosine is similar to that between *dl*-propranolol and l-epinephrine (Levitzki et al., 1976; Sevilla et al., 1976). After completion of activation experiments, tubes were transferred to 37 °C and a 20-min assay was performed. The activation of the enzyme by l-epinephrine and GppNHp was performed as previously described (Tolkovsky and Levitzki, 1978), under identical solution conditions used for the adenosine experiments.

Results

Activation of Adenylate Cyclase by Adenosine and GppNHp. The time course of activation by various concen-

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[1] Abbreviations used are: GppNHp, guanylyl imidodiphosphate; PEP, phosphoenolpyruvate; TME, 50 mM Tris-HCl buffer containing 2 mM $MgCl_2$ and 1 mM EDTA, pH 7.4; PPL, *dl*-PPL, propranolol; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid.

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TABLE II: Composition of PSL-IA, -IB, and -IC and of PSL-IIA, -IIB, and -IIC.[a]

	PSL (mol/mol of P)					
	IA	IB	IC	IIA	IIB	IIC
acetyl	1.1	0.99	0.97	0.02	0.03	0.08
long-chain base	1.0	1.0	1.1	1.1	1.0	1.0
inositol	1.0	0.91	0.96	0.73	0.99	1.2
hexuronic acid	0.87	0.92	0.96	0.84	1.2	1.2
hexosamine[b]	0.92	1.0	1.1	0.14	0.07	0.07
hexosamine[c]	1.1	1.1	1.1	0.86	1.1	1.2
total carbohydr[d]	4.5	5.6	6.4	5.0	4.9	5.9
pentose	1.7	3.1	3.8	2.6	2.1	2.7
arabinose	2.0	2.7	3.7	2.8	2.4	2.1
galactose	1.8	2.1	1.9	1.0	1.5	1.9
mannose						0.9

[a] Assay procedures for long-chain base, inositol, hexuronic acid, hexosamine, and total carbohydrate were the same as described earlier (Kaul and Lester, 1975). Acetyl, arabinose, and galactose assays were done by GLC as described under Materials and Methods. Mannose was assayed by the method of Lee (1972). Colorimetric pentose assay was as described by Dische and Borenfreund (1957). [b] 2 N HCl, 18 h, 100 °C. [c] 5.8 N HCl, 18 h, 100 °C. [d] Phenol-H_2SO_4.

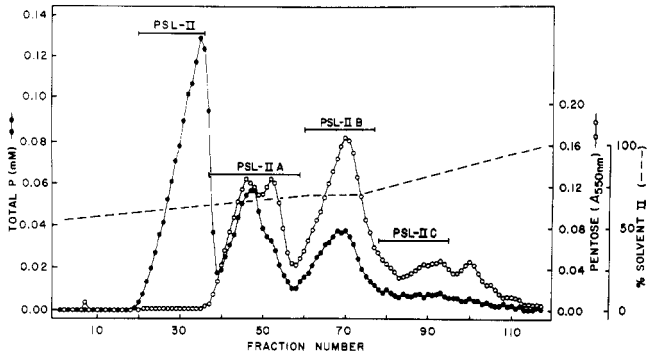

FIGURE 3: Column chromatography of fraction D_5 on Porasil. Fractions pooled for further purification are indicated. Other details are given under Materials and Methods (column III).

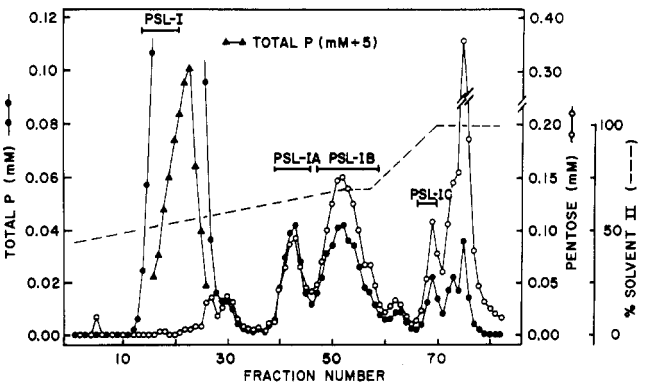

FIGURE 4: Column chromatography of fraction D_7 on Porasil. Fractions pooled for further purification are indicated. Other details are given under Materials and Methods (column I).

matographed on a 0.45 × 200 or 400 cm column of 37–75-μm Porasil, eluting with isocratic mixtures of chloroform, methanol, water, and ammonium hydroxide. The polarity of the eluting solvent was chosen such that the elution volume for the lipid sample was several times the void volume of the column. The column effluent was continuously monitored with a Pye Unicam moving wire detector which detects all nonvolatile carbon. All the purified lipids eluted off the Porasil column in one peak. All the fractions constituting the lipid peak were chromatographed on the above-mentioned TLC system. No inhomogeneity was observed. The purity of the nonacetyl lipids was further tested by *N*-acetylating them by the method of Roseman and Daffner (1956) followed by TLC of the product. Each of the three nonacetyl lipids gave a single product after N-acetylation with an R_f higher than the parent compound.

The phosphosphingolipids being reported in this paper are much less abundant than PSL-I and -II which together constitute about 40% of the total P in the phosphosphingolipid concentrate. The amounts of the purified lipids obtained, as a percent of the total P in the phosphosphingolipid concentrate, were: PSL-IA, 0.96%; PSL-IB, 0.27%; PSL-IC, 1.56%; PSL-IIA, 0.75%; PSL-IIB 3.9%; and PSL-IIC, 0.85%. It is difficult to precisely estimate the losses during purification but an educated guess would be about 50%. Therefore, these six lipids account for about 15–20% of the sphingolipid P.

Carbohydrate Composition of the Purified Phosphosphingolipids. As shown in Table II, all the phosphosphingolipids purified thus far contain 1 mol each of hexuronic acid, hexosamine, and inositol per mol P. In the PSL-II-like lipids from fraction D_5, the amino group of the hexosamine moiety is nonacetylated, and the hexosamine can only be hydrolyzed under strong hydrolytic conditions as previously observed with PSL-II (Kaul and Lester, 1975). On the other hand, the hexosamine moiety in the PSL-I-like lipids from fraction D_7 is N-acetylated as shown by the presence of one acetyl per P in these lipids and, as expected, the N-acetylated hexosamine could be hydrolyzed under conditions milder than those required for the nonacetylated sugar (Table II).

All of the purified lipids contained arabinose and galactose (Table II) as judged by GLC. Mannose was present in only PSL-IIC (Table II). With the exception of PSL-IIB, the arabinose and galactose data deviated less than 10% from integral values. For PSL-IIC, a higher value for pentose was obtained by the colorimetric method than by GLC. Whether these discrepancies can be attributed to experimental error or to undetected heterogeneity cannot be ascertained at this time. Presumably, detailed structural analysis experiments should resolve these questions. Carbohydrate analysis by GLC also revealed that the purified lipids did not contain any xylose, glucose, ribose, or fucose.

Long-Chain Base and Fatty Acid Composition of Purified Lipids. The long-chain base to P ratio in all lipids was 1:1 (Table II). Long-chain base and fatty acid fractions were obtained from CH_3OH–H_2O–HCl hydrolysis of lipids, as previously described (Kaul and Lester, 1975). Trimethylsilyl derivatives of the long-chain bases from all the lipids showed two peaks when analyzed by gas-liquid chromatography. These

TABLE III: Fatty Acid Composition of Purified Phosphosphingolipids.

PSL	% of detector response					
	OH:20	OH:22	OH:23	OH:24	OH:25	OH:26
IA	3.6	12.0	7.3	53.5	11.1	12.5
IB	3.5	10.4	6.7	57.6	10.4	11.4
IC	5.9	10.9	6.2	52.3	11.5	13.2
IIA	2.1	11.4	8.0	54.6	12.2	11.7
IIB	6.8	12.1	7.4	50.5	12.2	11.0
IIC	5.6	11.9	4.8	54.5	10.0	13.3

TABLE IV: Proposed Formulations of PSL-IA, -IB, and -IC and of PSL-IIA, -IIB, and -IIC.

PSL	proposed formulas	% phosphorus		
		found	calcd, anhydr	calcd, $3H_2O$
IA	PSL-I-[$(Ara)_2(Gal)_2$]	1.52	1.60	1.55
IB	PSL-I-[$(Ara)_3(Gal)_2$]	1.32	1.50	1.46
IC	PSL-I-[$(Ara)_4(Gal)_2$]	1.21	1.41	1.37
IIA	PSL-II-[$(Ara)_3(Gal)$]	*a*	1.68	1.63
IIB	PSL-II-[$(Ara)_3(Gal)_2$] or PSL-II-[$(Ara)_2(Gal)_2$]	1.50	1.54 or 1.65	1.50 or 1.61
IIC	PSL-II-[$(Ara)_2(Gal)_2(Man)$]	*a*	1.52	1.48

[a] Insufficient quantities available for estimation.

peaks had the same retention time as the ones obtained from PSL-I and -II, shown to be sphinganine and 4-D-hydroxy-8-sphingenine (Kaul and Lester, 1975). The long-chain base fraction obtained from all the lipids had the same R_f as authentic 4-D-hydroxysphinganine with TLC on silica gel developed with $CHCl_3$–CH_3OH–2 N NH_4OH (40:10:1), a system which does not resolve the two bases.

When chromatographed on thin-layer silica gel, the fatty acid methyl esters from all the lipids showed only one Rhodamine positive spot which corresponded to the methyl ester of a monohydroxy fatty acid. Gas chromatographic analysis of trimethylsilyl derivatives of the methyl esters showed the fatty acid composition of PSL-IA, -IB, and -IC and that of PSL-IIA, -IIB, and -IIC to be very similar to the fatty acid composition of PSL-I and -II. More than 50% of the total fatty acid content was the 2-hydroxy-C_{24} acid; C_{22}, C_{23}, C_{25}, and C_{26} 2-hydroxy acids were the less abundant components (Table III). The 2-hydroxy-C_{20} acid, present only in trace amounts in PSL-I and -II, comprised 2 to 6% of the total fatty acid content in these more complex lipids.

Formulation of PSL-IA, -IB, and -IC and PSL-IIA, -IIB, -IIC. Based on the experimental data presented, we propose the schematic formulas shown in Table IV for the six new tobacco phosphosphingolipids described in this paper. The percent phosphorus found in four of these lipids is consistent with these formulations.

Are the Nonacetylated Lipids Postharvest Artifacts? Since amino sugars in glycolipids and glycoproteins are generally thought to be N-acetylated, it is reasonable to question whether the PSL-II family is a postharvest artifact. In our original large-scale experiments involving 500 kg of fresh leaves, the leaves were put on ice and left for 4–5 h before further processing. We have attempted to see whether shortening this time results in any change in the ratio of nonacetylated to acetylated lipids. For this assay, we have taken advantage of the separation of the PSL-I and -II families by DEAE-cellulose chromatography (Figure 1 and Table I).

Crude extracts were prepared from leaves which were allowed to stand at room temperature for 5 h and for less than 10 min postharvest. These were compared by DEAE-cellulose chromatography (Figure 5) with the "standard" extraction conditions. The eluates were assayed for uronic acid which appears once in each PSL. This was judged to be a reasonably specific assay, since no other uronic acid containing lipids are known. It can be seen from Figure 5 that the ratios of the two major peaks were unchanged, suggesting that, insofar as the major PSL's are concerned, the appearance of nonacetyl lipids is not due to postharvest storage of the leaves prior to processing.

Lipid breakdown was observed in other experiments (unpublished), showing that if the leaves were homogenized in water and left for 5 h at room temperature we could recover less than one-third of the phosphosphingolipids; however, there was no indication of a conversion of the PSL-I family to the PSL-II family.

Discussion

We have presented evidence for the occurrence in tobacco leaves of two families of phosphosphingolipids whose separation from each other by anion-exchange chromatography is predicated on the charge difference arising from N-acetylation of an amino sugar. Three novel members of each family have been purified with a composition consistent with their being derivatives of PSL-I and -II, containing four to six additional neutral sugar residues, predominantly arabinose and galactose. These polar lipids, with two to three charged groups in addition to the seven to nine saccharide residues, posed difficult separation problems, e.g., requiring the use of 4-m columns. It can be seen from Figure 2 that there is overlap of R_f values in TLC between members of the two families, and these could probably not have been separated by silica gel adsorption chromatography. Different isolation methods will evidently have to be devised to purify the very polar phosphosphingolipids containing twice as many sugars (Figure 1 and Table I). As indicated under Results, perfectly integral values have not been obtained for the sugar composition of some of the substances (Table II). This may only reflect the vagaries of the analysis; however, establishing the purity of these substances will in some part rest on the degradation studies underway aimed at elucidating sugar linkages and anomeric configuration.

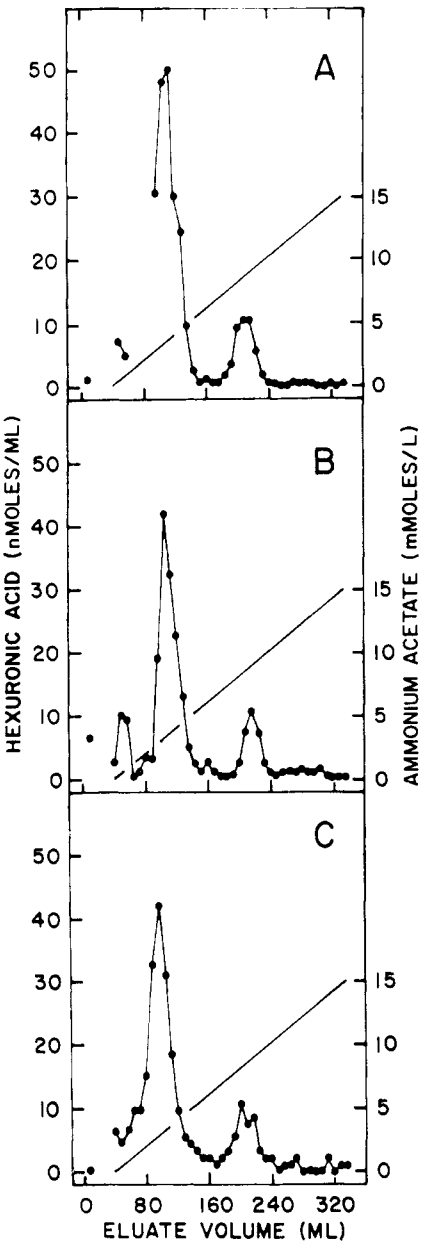

FIGURE 5: Assay of PSL-I and -II families of lipids by DEAE-cellulose column chromatography of lipid extracts from leaf homogenates prepared in three different ways. Homogenates of 2-kg batches of field-grown tobacco leaves were prepared by the following procedures: (A) leaves kept at room temperature for 5 h after harvest; (B) leaves kept at 5 °C for 5 h after harvest (Kaul and Lester, 1975); (C) leaves homogenized within 10-min postharvest. Subsequent steps of homogenization, extraction, and precipitation were as described earlier (Kaul and Lester, 1975). The phosphosphingolipid precipitate was washed with acetone and air dried. Approximately 2 g of the dried precipitate was taken up in 35 mL of pyridine–water (3:7) and mixed well with 30 mL (dry volume) of Hyflo Super Cel (Johns-Manville Co.) and 15 mL (dry volume) of Chelex 100 resin (Na^+, 100–200 mesh, Bio-Rad Laboratories). This mixture was filtered with suction through Whatman No. 1 filter paper and rinsed thrice with 25-mL portions of pyridine–water (3:7). The filtrates were combined. An 8-mL aliquot of the combined filtrates containing 11–20 μmol of phosphorus was put on a 0.95 × 15 cm acetate form DEAE-cellulose column. The column was eluted with 32 mL of methanol–water (85:15) followed by 304 mL of a linear gradient of 0.00 to 0.03 M ammonium acetate in $CHCl_3$–CH_3OH–H_2O–CH_3COOH (16:16:5:0.37). Hexuronic acid assay was done on 1-mL aliquots. All data have been normalized for a sample containing 12.6 μmol of P, the amount present in the sample for panel B. No data have been presented for certain fractions in A which gave a very high blank in the hexuronic acid assay.

The data so far obtained suggest that the presence of nonacetylated glucosamine is not artifactual. Attempts to change the ratio of acetylated to nonacetylated phosphosphingolipids by promoting autolysis have not been successful. These are, however, rather crude data (Figure 5), treating the phosphosphingolipids as two groups; this would not detect changes in the less abundant lipids if such existed. Since all other hexosamine-containing glyco conjugates are generally thought to be N-acetylated, it seems likely that the phosphosphingolipids are synthesized as the *N*-acetyl form with UDPGlcNAc as the GlcNAc donor. It will be worthwhile to examine whether the deacetylation which results in a membrane lipid of lowered negative charge reflects an interesting membrane regulatory mechanism.

The phosphosphingolipids we have isolated and partially characterized are evidently related to the "phytoglycolipids", the name given to a group of plant substances studied by H. E. Carter and co-workers and which occur in a number of seeds (Carter et al., 1958) and bean leaves (Carter and Koob, 1969). Their preparations were apparently heterogeneous, alkaline hydrolysis of corn phytoglycolipid yielding a mixture of oligosaccharides (Carter et al., 1964), with the predominant one, a tetrasaccharide, represented in the structure proposed by Carter et al. (1969):

glucosamine-(α1→4)glucuronic acid-
(α1→6)inositol-(2→1α)mannose
|
phosphoceramide

These workers have not dealt with the question of whether the hexosamine was originally free or N-acetylated. These lipids were obtained employing fairly harsh methods from commercial lipid preparations, including alkaline treatment to remove acyl esters. Stronger alkaline conditions were employed to obtain oligosaccharides for structural analysis. This treatment would have resulted in considerable deacetylation.

In contrast, our work indicates that, in the tobacco leaf, PSL-I and -II which contain no mannose are the major constituents. The low mannose content of the various DEAE-cellulose column fractions suggests that the tetrasaccharide form of "phytoglycolipid" discussed above is a minor component, if at all present. The only mannose-containing component thus far isolated, PSL-IIC, with an octasaccharide, constitutes about 0.85% of the total phosphosphingolipid concentrate. Carter and Kisic (1969) gave evidence for the occurrence of arabinose in hydrolysates of phytoglycolipid preparations. Our work establishes the presence of arabinose for the first time in highly purified lipids. Arabinogalactans are well established components of plant cell walls (Albersheim, 1976). It should be interesting to find out if the linkages and anomeric configuration of the carbohydrates in the arabinose- and galactose-containing oligosaccharides are similar to those in the cell-wall arabinogalactans.

Since phytoglycolipid referred to a group of substances, we prefer the PSL (phosphosphingolipid) nomenclature with each component specifically designated, PSL-I, PSL-IA, etc. It is not possible to have a completely rational system of nomenclature until detailed structures of most of the phosphosphingolipids have been worked out. In addition to the eight phosphosphingolipids so far purified, there may be roughly a dozen more components in the concentrate from tobacco leaves not yet purified, judging from chromatography on Porasil and by TLC.

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Structure of a Major Glycophosphoceramide from Tobacco Leaves, PSL-I: 2-Deoxy-2-acetamido-D-glucopyranosyl(α1→4)-D-glucuronopyranosyl(α1→2)myoinositol-1-*O*-phosphoceramide†

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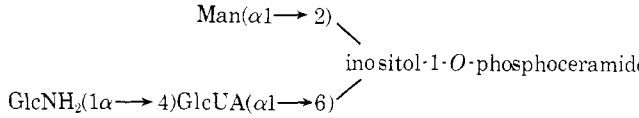
ABSTRACT: The chemical structure of a major glycophosphoceramide from tobacco leaves, called PSL-I [K. Kaul and R. L. Lester (1975), *Plant Physiol. 55,* 120], has now been characterized as 2-deoxy-2-acetamido-D-glucopyranosyl-(α1→4)D-glucuronopyranosyl(α1→2)myoinositol-1-*O*-phosphoceramide. Sites of glycosidic linkage were determined by (1) methylation analysis on a trisaccharide isolated by degradation of carboxyl-reduced PSL-I and (2) periodate oxidation experiments on PSL-I. The resulting products were identified with gas chromatography/mass spectrometry. Anomeric configurations were determined by resistance of the sugars in the peracetylated trisaccharide to chromium trioxide treatment.

A class of complex glycophosphoceramides containing phosphoinositol occurs in higher plants and fungi (Carter et al., 1965; Lester et al., 1974). These lipids may be analogous to the glycolipids found in animal cell plasma membrane, which in the animal cells carry ABO, Lewis and Forssmann antigen activities, among many others (Hakomori, 1970; Hakomori et al., 1972; Siddiqui and Hakomori, 1971), as well as receptors for cholera toxin (Cuatrecasas, 1973; Holmgren et al., 1975), clostridial toxins (Van Heyningen and Miller, 1961; Berheimer and Van Heyningen, 1961; Van Heyningen, 1974), hormones (Mullin et al., 1978), and perhaps viruses (McCrea, 1953; Klenk and Uhlenbrook, 1958; Kathan et al., 1959; Kathan and Winzler, 1963). Finding the true function of glycolipids in either plants or animals is a challenging research goal.

Carter and his co-workers (Carter et al., 1958, 1962; Carter and Koob, 1969) were the first to study phosphoinositol-containing glycophosphoceramides prepared from a variety of plant materials such as commercial vegetable oil, corn and flax seeds, and leaves of bush beans. Apparently, homogeneous glycolipids were not obtained, since alkaline hydrolysis of these preparations yielded a mixture of oligosaccharides, suggesting the occurrence of a diverse series of glycolipids in plants (Carter and Kisic, 1969). The major oligosaccharide from corn seed was isolated in large quantity and was chemically characterized leading to the following structural proposal for corn "phytoglycolipid" (Carter et al., 1969):



The isolation procedures employed by Carter and his coworkers involved treatment with alkali to remove acyl esters (Carter et al., 1958) or refluxing with 70% ethanol containing 0.1 N HCl (Carter and Koob, 1969).

To preclude possible breakdown of labile bonds in the glycolipids, a relatively mild extraction procedure was devised for the preparation of a glycophosphoceramide concentrate from fresh tobacco leaves, yielding about 100 μmol of P/kg fresh weight (Kaul and Lester, 1975). Eight components have been

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