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Lipophosphonoglycan of the Plasma Membrane of *Acanthamoeba castellanii*

INOSITOL AND PHYTOSPHINGOSINE CONTENT AND GENERAL STRUCTURAL FEATURES

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Lipophosphonoglycan, a major component of the plasma membrane of *Acanthamoeba castellanii*, has now been shown to contain 8% inositol and 13% C_{25}- and C_{24}-phytosphingosines in addition to the previously identified content of neutral sugars (26%), amino sugars (3%), aminophosphonates (10%), acid-hydrolyzable phosphate (3%), and long chain fatty acids (14%). The fatty acids and phytosphingosines are in ceramide groups. Lipophosphonoglycan can be separated by dodecyl sulfate-polyacrylamide electrophoresis into two major components that are similar in composition except for different oligosaccharide groups. A tentative structural model incorporating these features is proposed in which each of the two components of lipophosphonoglycan is conceived as an oligomeric inositol-containing glycosphingolipid.

Highly purified plasma membranes from the soil amoeba, *Acanthamoeba castellanii*, contain (1, 2) approximately 25% phospholipid, 13% sterol, 35% protein, and 29% lipophosphonoglycan. Since this amoeba does not have a cell wall or any other detectable material external to the plasma membrane (3) and since electron microscopic cytochemistry (4) demonstrates lipophosphonoglycan at both surfaces of the plasma membrane, it is most probable that this novel polymer is an integral component of the membrane bilayer.

Lipophosphonoglycan, purified from whole amoebae (5), had previously been shown to contain 26% neutral sugars (glucose/mannose/galactose/xylose; mole ratio approximately 5/4/1/1), 3.3% amino sugars (galactosamine/glucosamine; mole ratio 3/1), 10% aminophosphonates (2-aminoethylphosphonate/1-hydroxy-2-aminoethylphosphonate; mole ratio 1/1), 3.2% acid-hydrolyzable phosphate, and 14% long chain fatty acids, accounting for 57% of the total mass. The fatty acids are distinctly different from the fatty acids of the plasma membrane phospholipids and include (2) three classes of odd and even number compounds: (*a*) 33% saturated and unsaturated, normal and branched C_{16}–C_{28} fatty acids; (*b*) 61% normal and branched, saturated 2-hydroxy C_{22}–C_{28} fatty acids; (*c*) 6% tentatively identified as normal and branched, saturated 2-hydroxy-3-methyl fatty acids.

We have now accounted for an additional 21% of the mass of lipophosphonoglycan as inositol and long chain phytosphingosine bases. Analyses of the lipid-soluble products of partial acid and alkaline hydrolyses indicate that the phytosphingosines are all in ceramide groups. Additional information on the organization of the rest of the polymer was provided by analyses of the water-soluble products of partial acid and alkaline hydrolyses and of the two components of lipophosphonoglycan separated by dodecyl sulfate-polyacrylamide gel electrophoresis.

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EXPERIMENTAL PROCEDURES

Acid and Alkaline Hydrolysis—Lipophosphonoglycan (5), 5 to 10 mg/ml, was hydrolyzed in nitrogen-flushed, evacuated, sealed tubes in HCl or NaOH as indicated. Hydrolysates were lyophilized (after acidifying the alkaline hydrolysates) and the residues were suspended in chloroform/methanol, 2/1. After recovery of insoluble material by centrifugation, the chloroform/methanol solutions were separated by addition of 0.2 volume of water into organic and aqueous phases which were washed twice with clean, opposite phase to give a "chloroform-soluble" fraction and a "water-soluble" fraction. The "chloroform/methanol-insoluble" fraction from partial acid hydrolysis was soluble in 0.1 N NH_4OH and the insoluble fraction from partial alkaline hydrolysis was soluble in 0.1 M pyridinium acetate, pH 7.

Hydroxylaminolysis—The method of Snyder and Stephens (6) was modified to allow solubility of lipophosphonoglycan in the reaction mixture. Lipophosphonoglycan was dissolved in 8% NaOH (2 mg/0.5 ml) and 0.25 ml of water and 0.25 ml of 8% hydroxylamine-HCl in 95% ethanol were added. The solution was heated at 65° for up to 60 min, cooled, and lyophilized. The dried material was suspended in 0.5 ml of 2 N formic acid to which was added 9.5 ml of chloroform/methanol, 2/1 and, after vigorous mixing, 2 ml of water. The aqueous and organic phases were washed with clean opposite phases and the pooled organic phases were taken to dryness under a stream of nitrogen.

Ammonolysis—Lipophosphonoglycan was heated in concentrated NH_4OH (17 M) in sealed, evacuated tubes at 100° for various times up to 18 hours. The NH_4OH was removed by lyophilization and the residue fractionated as described above into a chloroform/methanol-insoluble fraction, chloroform-soluble fraction, and water-soluble fraction.

Gel Electrophoresis—Analytical and preparative dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (7) using 14% gels. All samples were heated in a boiling water

bath for 10 min in the sample buffer before applying them to the gels. Gels were stained for carbohydrate with the periodic acid-Schiff reagent (1). Lipophosphonoglycan was extracted from appropriate regions of preparative gels by distilled water, dialyzed extensively, and lyophilized.

Analyses—Fractions to be analyzed for free fatty acids were dissolved in chloroform, methylated with diazomethane, and the fatty acid methyl esters identified and quantified by gas chromatography on 3% OV-17 (2). For total fatty acid analysis, fractions were hydrolyzed in 4 N HCl at 100° for 4 hours and the free fatty acids were extracted into chloroform, methylated, and analyzed.

Total phosphorus was analyzed by the method of Ames and Dubin (8) and inorganic phosphate by the same procedure without fusion with $Mg(NO_3)_2$. Total carbohydrate was measured by the phenol-sulfuric acid method (9) and reducing groups were determined by the method of Park and Johnson (10) with glucose as the standard. Individual neutral sugars were identified and quantified by gas chromatography on 3% OV 225 (Supelco Inc., Bellefonte, Pa.) as the peracetylated derivatives of the polyols formed by reduction with $NaBH_4$ (11). Amino sugars were analyzed on the Beckman 121 amino acid analyzer using the extended short column (12). For total neutral sugar and glucosamine analyses, fractions were hydrolyzed in 2 N HCl at 105° for 2 hours and for galactosamine content fractions were hydrolyzed in 4 N HCl at 105° for 16 hours, these being the optimal conditions established previously (5). Acid-hydrolyzable phosphate and aminophosphonates were measured as inorganic phosphate and organic phosphate after hydrolysis in 6 N HCl at 105° for 16 hours (5).

Inositol was identified and quantified in the water-soluble fractions of acid hydrolysates after removal of the HCl by lyophilization in two ways. It was converted to its per(trimethylsilyl) derivative by heating dried samples in dry pyridine/hexamethyldisilazane/trichloromethylsilane, 5/2/1 (Applied Science Laboratories, State College, Pa.) at 120° for 30 min and analyzed by gas chromatography on 3% OV-17 and by mass spectrometry using an LKB mass spectrometer, at a nominal ionization voltage of 70 eV, equipped with a gas chromatographic inlet containing 1% OV-17. Inositol was also analyzed by inositol dehydrogenase (Sigma Chemical Co., St. Louis, Mo.) according to the procedure of Kaul and Lester (13).

Glycerol was analyzed by glycerol dehydrogenase (Sigma Chemical Co., St. Louis, Mo.), as its Tris(trimethylsilyl) derivative by gas chromatography-mass spectrometry and by periodate oxidation followed by formaldehyde assay by chromotropic acid (14). For glycerol analysis, acid hydrolysates were neutralized by passage through Dowex 1 bicarbonate.

Neuraminic acid was analyzed by gas chromatography on 3% OV-17 as its *N*-acetyl trimethylsilyl derivative (15). For this analysis lipophosphonoglycan was methanolyzed by methane sulfonic acid (16), dried under a stream of nitrogen, and the water-soluble fraction recovered by partitioning in a chloroform/methanol/water system.

Long chain bases were extracted from alkaline or acid hydrolysates of lipophosphonoglycan by the addition of 19 volumes of chloroform/methanol, 2/1 (acid hydrolysates were first adjusted to pH greater than 11) followed by 0.2 volume of water to separate the phases. Long chain bases were quantified by the methyl orange method of Lauter and Trams (17) and identified by thin layer chromatography on 5-mm thick plates of Silica Gel H, containing 1.95% potassium oxalate, developed with a solvent of chloroform/methanol/2 N NH_4OH, 40/15/1. Bands were detected by exposure to iodine vapor or by spraying with 0.2% ninhydrin in acetone/pyridine, 9/1, followed by heating at 110° for 5 to 10 min. Long chain bases were converted to their per(trimethylsilyl) derivatives by reaction with bis(trimethylsilyl) trifluoroacetamide in a 2/1 mixture with dry pyridine at room temperature for 10 min; to their *N*-acetyl trimethylsilyl derivatives by reacting with anhydrous methanol/acetic anhydride, 1/1, at room temperature, adding a drop of water, lyophilizing, and then reacting with the silylating reagents as before; to their deuterium$_9$-trimethylsilyl derivatives by treating in a similar manner with D_9-trimethylsilyl-imidazole; and were subjected to periodic acid cleavage by the CS_2 method of Karlsson *et al.* (18). All the derivatives and the aldehydes produced by periodate cleavage were identified by gas chromatography on 3% OV-17 combined with mass spectrometry. Authentic sphingosine, dihydrosphingosine, and C_{18}-phytosphingosine and the reagents were obtained from Applied Science Laboratories (State College, Pa.) and Supelco, Inc. (Bellefonte, Pa.).

Monoesterified Phosphate—Lipophosphonoglycan and hydrolysate fractions were incubated with *Escherichia coli* alkaline phosphatase (19) (Worthington Biochemical Co., Freehold, N. J.), specific activity: 17 μmol of *p*-nitrophenylphosphate hydrolyzed/min/mg of protein. Fractions containing 1 to 2.5 μmol of acid-hydrolyzable phosphate were incubated with 1 unit of alkaline phosphatase at 28° for 1 hour (24- to 60-fold excess) in 0.5 M Tris-HCl, pH 8.0, and total and inorganic phosphate were determined at the end of the reaction. Lipophosphonoglycan was also incubated with enzyme in 0.5 M Tris-HCl, pH 8.0, saturated with 1-butanol and 0.5 M Tris-HCl, pH 8.0, containing 0.1% sodium dodecyl sulfate. Alkaline phosphatase activity was unaffected by the 1-butanol and 10% greater in the presence of dodecyl sulfate.

RESULTS

Identification of Inositol—Inositol was identified in the water-soluble fraction of acid hydrolysates of lipophosphonoglycan as its trimethylsilyl derivative. A major gas chromatographic peak had the same retention time as authentic hexa(trimethylsilyl)myo-inositol and the same mass spectrum with a molecular ion $(M)^+$ of *m/e* 612 and major fragments at *m/e* 432, 318, 305, and 217. The presence of inositol was confirmed by the assay using inositol dehydrogenase. Maximum yields of inositol were obtained after hydrolysis of lipophosphonoglycan in 4 N HCl at 105° for 16 hours. In separate experiments with different batches of lipophosphonoglycan, 0.2 μmol (gas chromatographic assay) and 0.35 μmol (inositol dehydrogenase assay) were recovered from 1 mg of lipophosphonoglycan. The enzymatic assay is considered more reliable.

Neuraminic acid was not detected in lipophosphonoglycan. Less than 0.05 μmol of glycerol/mg of lipophosphonoglycan was detected by any of the three assays. It is possible that this very small amount of glycerol is derived from contaminating phospholipids.

Identification of Lipid Bases—Thin layer chromatography of the chloroform-soluble fraction of acid hydrolysates of lipophosphonoglycan extracted at alkaline pH revealed an iodine and ninhydrin-positive component with an R_F slightly greater but overlapping that of C_{18}-phytosphingosine. Gas chromatography on 3% OV-17 of the per(trimethylsilyl) derivative of this fraction gave a major peak with a retention time 5.6 times longer than the retention time of the trimethylsilyl derivative of C_{18}-phytosphingosine (22.4 min *versus* 4 min). The mass spectrum of the derivative of the unknown lipid base (Fig. 1*A*) was qualitatively similar to the mass spectrum of trimethylsilyl C_{18}-phytosphingosine, differing only in that the molecular ion and the larger fragments each had masses greater by 98 *m/e* units than the corresponding ions from the standard. This indicates that the major unknown lipid base is a C_{25}-phytosphingosine. Similarly, a minor peak (at 20.4 min) on the gas chromatogram of the per(trimethylsilyl) derivatives of the chloroform-soluble fraction had a mass spectrum consistent with its identification as C_{24}-phytosphingosine. These structural assignments were supported by the gas chromatographic-mass spectral analyses of the products of periodate oxidation of the chloroform-soluble fraction. A major peak had the mass spectrum of a C_{22}-aldehyde (Fig. 1*B*) and a minor peak could be identified as a C_{21}-aldehyde.

The structures of the C_{25}- and C_{24}-phytosphingosines were also confirmed by the mass spectra of their *N*-acetyl trimethylsilyl derivatives which were identical with the mass spectrum of the corresponding derivative of authentic C_{18}-phytosphingosine except for the expected differences in the masses of the larger ions. All three spectra had major peaks at mass *M*-103, *M*-174, and *M*-276 (20). The last fragment, *M*-276, is derived from cleavage between C-3 and C-4, a cleavage unique to phytosphingosines. Further confirmation of the identities of

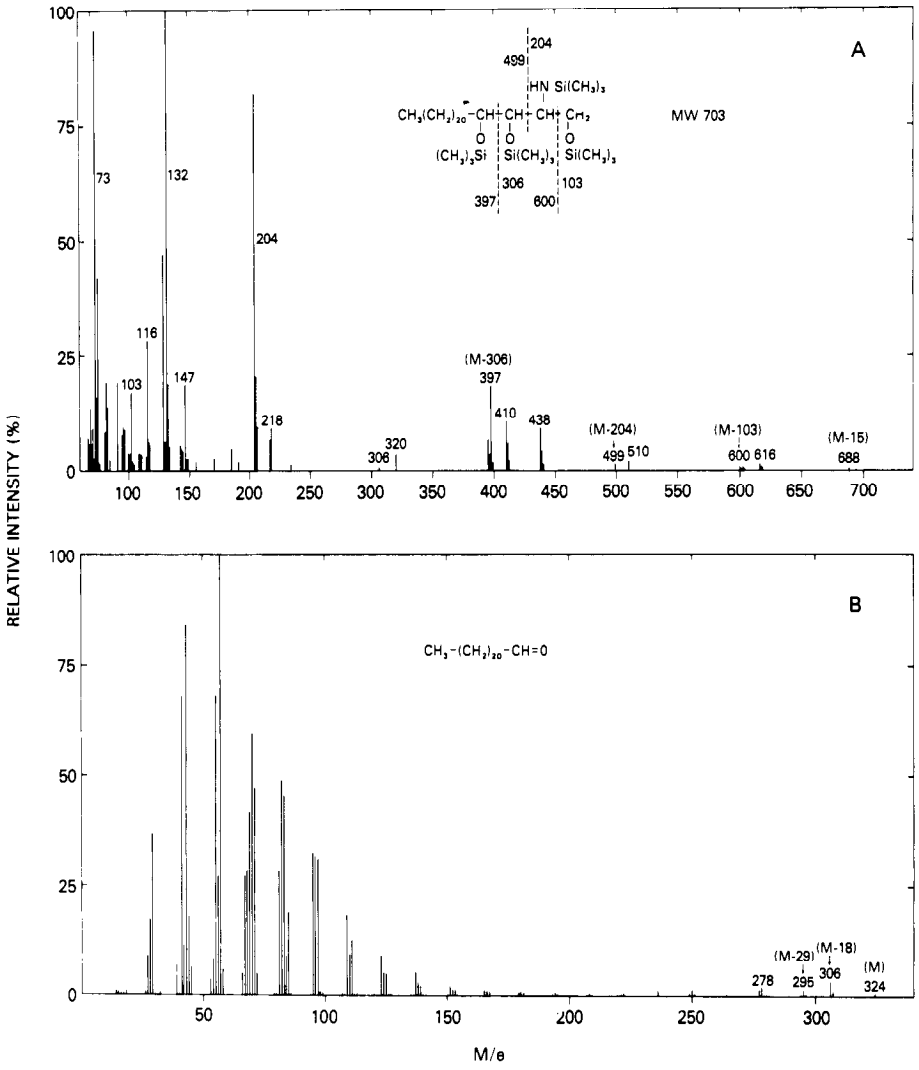

FIG. 1. Mass spectrum of the per-(trimethylsilyl) derivative of the major phytosphingosine base isolated from acid and alkaline hydrolysates of lipophosphonoglycan (*A*) and of the aldehyde produced by periodate oxidation of the phytosphingosine (*B*). The formulas illustrate the probable origins of the fragments but the data do not distinguish between a linear or branched structure. From their resolution times relative to C_{18}-phytosphingosine it is possible that the C_{24}-base is linear and the C_{25}-base is branched.

the C_{25}- and C_{24}-phytosphingosines was obtained by the mass spectra of the derivatives formed with deuterotrimethylsilyl imidazole which gave unequivocal evidence for four trimethylsilyl groups on each of the long chain bases.

The C_{25}- and C_{24}-phytosphingosines were released in higher yield by alkaline hydrolysis than by acid hydrolysis. Maximum yield, 0.30 μmol of phytosphingosine/mg of lipophosphonoglycan, was obtained after hydrolysis for 42 hours in 1 N NaOH; lower yields were obtained when the alkaline hydrolysis was extended longer than 42 hours. The C_{24}-phytosphingosine was 6% of the total.

Hydroxylaminolysis and Ammonolysis of Lipophosphonoglycan—Lipophosphonoglycan contains approximately 14% by weight of long chain fatty acids (2). Hydroxylaminolysis for 1 hour released 21% of the palmitate and 8% of the oleate and ammonolysis for 18 hours released 64% of the palmitate and 18% of the oleate but no detectable amounts of the other 28 different fatty acids of lipophosphonoglycan. The remainder of the fatty acids were released as free fatty acids by hydrolysis in 4 N HCl at 100° for 4 hours (2) of the material resistant to hydroxylaminolysis and ammonolysis. Since palmitate and oleate account for only 2.5% and 10%, respectively, of the total fatty acids of lipophosphonoglycan (2), it seems likely that most of the fatty acids are in amide linkages resistant to cleavage by hydroxylamine and ammonia.

Identification of Ceramides—Lipophosphonoglycan was hydrolyzed in 3 ml of 1 N NaOH for 4 hours at 100° or in 10% NH_4OH (v/v) for 16 hours at 155°, and the hydrolysate extracted by adding 5 ml of isopropyl alcohol/heptane, 4/1, followed by 3 ml of heptane. The organic phase was washed with clean alkaline aqueous phase and twice with acidic aqueous phase and then analyzed by thin layer chromatography on silicic acid, developed by chloroform/methanol, 1/1. Two bands were detected by iodine vapor and both were positive in the benzidine spray for amides (21). The major band had an R_F of 0.08, the minor band an R_F of 0.20, authentic fatty acyl sphingosine (Supelco, Inc., Bellefonte, Pa.) an R_F of 0.37, and authentic hydroxy fatty acyl sphingosine (Supelco, Inc.) an R_F of 0.21.

The bands were eluted from the silicic acid with chloroform/methanol/water, 25/15/2.5 (2) and methanolyzed by heating in 2 N methanesulfonic acid in anhydrous methanol for 16 hours at 65°. One volume of water and 2 volumes of ethyl acetate were added and the phytosphingosine bases were recovered from the aqueous phase after making it alkaline, derivatized, and shown to be identical with the C_{25}- and C_{24}-phytosphingosines previously described. Both bases were present in both thin layer chromatographic fractions. The fatty acid methyl esters were recovered from the organic phase and analyzed by gas chromatography on 3% OV-17. As anticipated the fatty

acids recovered from the major thin layer chromatographic band (lower R_F) included all of the 2-hydroxy and branched 2-hydroxy fatty acid present in lipophosphonoglycan and the nonhydroxy fatty acids were recovered from the minor thin layer chromatographic band (higher R_F) (Table I). Palmitate and oleate were present in both fractions.

Products of Partial Alkaline Hydrolysis—Lipophosphonoglycan was hydrolyzed in 1 N NaOH at 105° for 4 hours and the hydrolysate fractionated into a chloroform/methanol/water-insoluble fraction, a chloroform-soluble fraction, and a water-soluble fraction. The chloroform/methanol/water-insoluble fraction contained 80% of the total phosphorus and essentially all of the neutral sugars of the lipophosphonoglycan. There were no detectable reducing groups. This fraction was separated on Sephadex G-50 (Fig. 2) into four peaks, the compositions of which are compared to the composition of lipophosphonoglycan in Table II. Fraction D, which contained no sugars, had a ratio of acid-hydrolyzable phosphate/aminophosphonates/inositol similar to that of the intact polymer. Fractions B and C contained varying amounts of most of the constituents of lipophosphonoglycan except that Fraction B contained no xylose and Fraction C contained no galactose.

TABLE I

Fatty acid composition of ceramides

The major and minor ceramides produced by partial alkaline hydrolysis of lipophosphonoglycan were separated by thin layer chromatography and their fatty acid compositions determined as described in the text. The data are compared to those previously obtained (2) for a different batch of lipophosphonoglycan. The values for the major ceramide are normalized to branched, 2-hydroxy 22:0 and the values for the minor ceramide are normalized to branched 22:0. Palmitate and oleate were present in both ceramide fractions and are not reported. The other fatty acids were exclusively in one or the other ceramide.

Fatty acid	Lipophosphonoglycan	Minor ceramide	Major ceramide
Saturated			
22:0	0.18	0.37	0
25:0	0.47	0.49	0
26:0	3.24	3.63	0
27:0	1.71	1.19	0
28:0	2.76	1.21	0
Branched saturated			
21:0	—[a]	0.42	0
22:0	1.00	1.00	0
23:0	1.88	1.64	0
24:0	1.71	1.26	0
25:0	0.88	0.52	0
2-Hydroxy			
22:0	—[a]	0	0.17
25:0	0.45	0	0.23
26:0	3.14	0	2.65
27:0	2.23	0	1.02
28:0	1.92	0	0.64
Branched 2-hydroxy			
21:0	—[a]	0	1.43
22:0	1.00	0	1.00
23:0	1.62	0	2.48
24:0	1.08	0	1.54
25:0	0.39	0	0.35

[a] These were minor constituents of the lipophosphonoglycan originally analyzed (2) and were ignored; they were relatively more major components of the ceramides derived from a different preparation of lipophosphonoglycan.

Fraction A provided too little material to carry out all of the analyses. Of the total acid-hydrolyzable phosphate in Fractions B, C, and D, 23%, 18%, and 67%, respectively, were hydrolyzable by alkaline phosphatase (Table III), whereas only about 7% of the phosphate of intact lipophosphonoglycan was released by the enzyme and that only in the presence of sodium dodecyl sulfate (Table III).

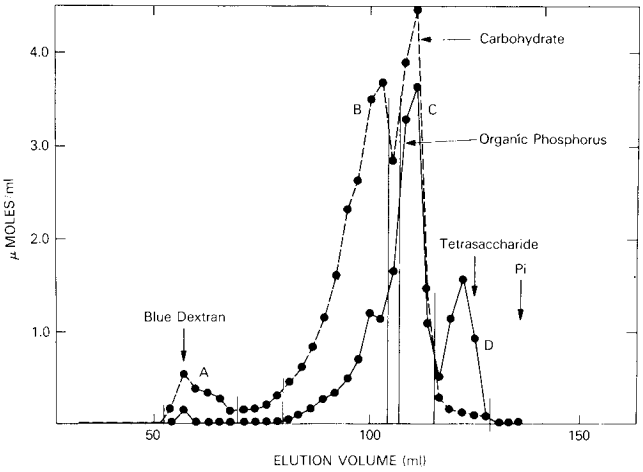

FIG. 2. Chromatography of Sephadex G-50 of the chloroform/methanol/water-insoluble fraction obtained from hydrolysis of lipophosphonoglycan in 1 N NaOH for 4 hours at 105°. The fraction was dissolved and chromatographed in 0.1 N pyridinium acetate, pH 7.0. This fraction contained 80% of the total phosphorus and 95% of the total carbohydrate of the starting material. Fractions A to D contained 6%, 62%, 32%, and a trace of the total carbohydrate applied to the column. Their total analyses are shown in Table II. The elution positions of blue dextran, Stachyose (tetrasaccharide), and inorganic phosphate (*Pi*) are shown by the *vertical arrows*.

TABLE II

Analysis of products of partial alkaline hydrolysis of lipophosphonoglycan

The chloroform/methanol-insoluble fraction after hydrolysis of lipophosphonoglycan in 1 N NaOH for 4 hours at 105° contained 80% of the total phosphorus and 95% of the carbohydrate. It was dissolved in 0.1 M pyridinium acetate and separated on Sephadex G-50 into four fractions (*A* to *D*; Fig. 2) which contained 6%, 62%, 32%, and a trace of the total carbohydrate, respectively. The fractions were then hydrolyzed under the appropriate conditions to get maximal yeilds of each of the constitutents, the analysis of which is reported in the table.

	LPG[a]	A	B	C	D
	μmol/μmol total phosphorus				
Glucose	0.56	4.48	1.94	0.13	
Mannose	0.45	0	0.91	0.84	
Galactose	0.12	2.41	0.38	0	
Xylose	0.12	0	0	0.20	
Glucosamine	0.04		Trace	Trace	Trace
Galactosamine	0.11		0.04	0.10	Trace
Acid-hydrolyzable phosphate	0.31		0.21	0.37	0.25
Aminophosphonates	0.69		0.79	0.63	0.75
Inositol	0.15 (0.25)[b]		0.12	0.24	0.12

[a] Data for lipophosphonoglycan (LPG) are from Ref. 5 except for the inositol value.

[b] Inositol content is 0.25 μmol/μmol of phosphorus H total by enzymatic assay and 0.15 by gas chromatography. The inositol content of the partial hydrolysis products was determined by gas chromatography.

TABLE III

Inorganic phosphate released by alkaline phosphatase

Lipophosphonoglycan and several fractions, described in Figs. 2 and 3 and Tables II and IV, derived from partial alkaline and acid hydrolysis were incubated with alkaline phosphatase as described under "Experimental Procedures" and the release of inorganic phosphate was measured.

Substrate	Acid-hydrolyzable phosphate release
	% of total
Lipophosphonoglycan	
Aqueous buffer	<1
+ 1-butanol	<1
+0.1% sodium dodecyl sulfate	7.5
Alkaline hydrolysis fractions (Table II, Fig. 2)	
Fraction B	23
Fraction C	17.5
Fraction D	66.7
Acid hydrolysis fractions (Table IV, Fig. 3)	
Fraction B	31.2
Insoluble	15.2

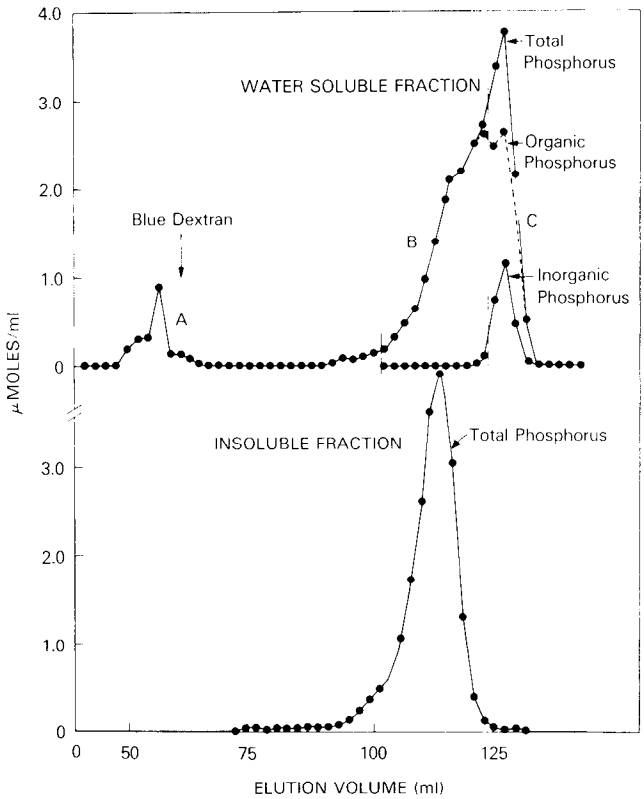

FIG. 3. Chromatography on Sephadex G-50 of the water-soluble fraction (65% of the total phosphorus) and the chloroform/methanol/water-insoluble fraction (35% of total phosphorus) obtained from hydrolysis of lipophosphonoglycan in 2 N HCl for 4 hours at 105°. The total analyses of these fractions are shown in Table IV.

The water-soluble fraction obtained by fractionation of the partial alkaline hydrolysis contained too little material to examine further.

Products of Partial Acid Hydrolysis—Lipophosphonoglycan was hydrolyzed in 2 N HCl for 4 hours at 105°, conditions known to release the neutral sugars, glucosamine, and most of the lipids as monomers but to leave the other constituents mostly in bound form (2, 5). The hydrolysate was fractionated into a chloroform/methanol/water-insoluble fraction which contained 35% of the total phosphorus, a water-soluble fraction that contained 65% of the phosphorus, and a chloroform-soluble fraction that contained the fatty acids and long chain bases. The water-soluble fraction was separated on Sephadex G-50 (Fig. 3) into a minor voided Fraction A and overlapping Fractions B and C (the latter at the salt boundary). The chloroform/methanol/water-insoluble material readily dissolved in 0.1 N NH_4OH and gave one peak on Sephadex G-50 at the approximate position of water-soluble Fraction B (Fig. 3). The compositions of water-soluble Fraction B and the chloroform/methanol/water-insoluble material were not very different from the composition of lipophosphonoglycan with respect to galactosamine, acid-hydrolyzable phosphate, aminophosphonates, and inositol (Table IV). Alkaline phosphatase hydrolyzed 31% and 15%, respectively, of the acid-hydrolyzable phosphate of Fraction B and the chloroform/methanol/water-insoluble fraction (Table III). Water-soluble Fraction C contained a compound tentatively identified as inositol phosphate by gas chromatographic-mass spectral analysis of their per(trimethylsilyl) derivatives.

TABLE IV

Analysis of products of partial acid hydrolysis

Compositions of water-soluble Fractions A and B (Fig. 5) and chloroform/methanol/water-insoluble fraction (Fig. 5) obtained by chromatography on Sephadex G-50 of the products of hydrolysis of lipophosphonoglycan (LPG) in 2 N HCl, 105° for 4 hours are compared to the composition of the starting material.

	LPG[a]	Water-soluble		Insoluble
		A	B	
	μmol/μmol total phosphorus			
Galactosamine	0.11	0.05	0.06	0.12
Phosphate	0.31	0.71	0.40	0.25
Aminophosphonates	0.69	0.29	0.60	0.75
Inositol	0.15[b]	Trace	0.26	0.16

[a] See Table II, Footnote *a*.

[b] See Table II, Footnote *b*.

Analysis of Fractions Separated by Dodecyl Sulfate Gel Electrophoresis—Dodecyl sulfate-polyacrylamide gel electrophoresis separates lipophosphonoglycan into two bands of high mobility (1) which, when isolated by preparative gel electrophoresis, each give a single band with its original mobility (Fig. 4). Gel scans (3) showed equal intensity of stain in the two components of lipophosphonoglycan while the isolated faster component contained 90% of the stainable carbohydrate on its gel and the isolated slower component accounted for 97% of the stainable carbohydrate on its gel. Both the fast and slow components had the same ratios of acid-hydrolyzable phosphate to total phosphate and of inositol to total phosphate as the original lipophosphonoglycan. By qualitative analysis of appropriate hydrolysates on thin layer chromatograms, both fast and slow components contained phytosphingosines and hydroxy and nonhydroxy fatty acids. The components differed significantly, however, in their carbohydrate content and in the relative ratios of the individual sugars (Table V). The fast component contained almost no galactose and the slow component contained no xylose. These sugar compositions are very similar to those obtained in Fractions C and B, respectively,

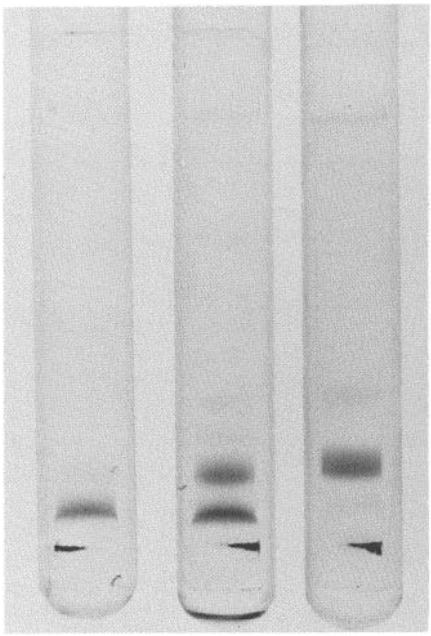

FIG. 4. Dodecyl sulfate-polyacrylamide gel electrophoretic analysis of lipophosphonoglycan fractionated by preparative gel electrophoresis. The *middle gel* is the unfractionated lipophosphonoglycan; the *left gel* is the faster component; and the *right gel* is the slower component. Gels were stained with periodic acid-Schiff reagent. The position of bromphenol blue is marked.

TABLE V

Sugar analysis of electrophoretically separated components of lipophosphonoglycan

	Fast component	Slow component	Fast + slow calculated[a]	LPG[b]
	μmol/μmol phosphorus total			
Total sugars	0.93	1.9	1.3	1.25
Glucose	0.18	1.18	0.58	0.56
Mannose	0.47	0.57	0.51	0.45
Galactose	0.04	0.15	0.10	0.12
Xylose	0.24	0	0.10	0.12

[a] Calculated assuming that 60% of the phosphorus total of lipophosphonoglycan is in the fast component (1).

[b] From Ref. 5.

from partial alkaline hydrolysates of intact lipophosphonoglycan (Table II).

DISCUSSION

Addition of approximately 8% inositol (enzymatic assay) and 13% long chain phytosphingosines to the previously identified components accounts for about 80% of the mass of lipophosphonoglycan. Some of the missing 20% is probably contributed by water and counter ions and perhaps by underestimation of some of the known constituents owing to losses during hydrolysis and analysis. Our current working models of the general structures of the two electrophoretically separable components of lipophosphonoglycan are illustrated in Fig. 5. Glucosamine is omitted from the models because it has not been found in any of the products of partial hydrolysis.

The near equivalence of the molar ratio of long chain fatty acids and bases (0.33 and 0.3 μmol/mg of lipophosphonoglycan, respectively; 0.25 and 0.23 μmol/μmol of phosphorus total), the stability of most of the fatty acid linkages to reagents that

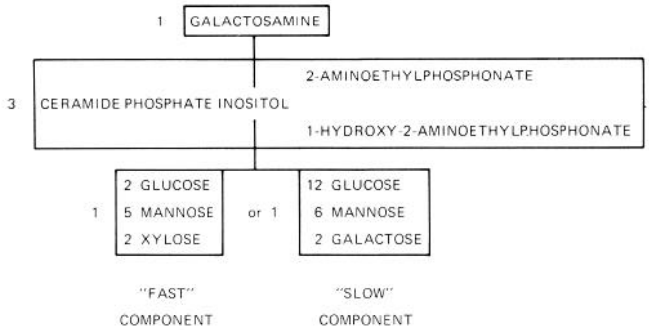

FIG. 5. Working model for the structure of the two components of lipophosphonoglycan. Glucosamine is not shown because it has not been identified in the products of partial hydrolysis. The molar stoichiometry of the constituents is calculated from data in this and previous (2, 5) papers. Constituents within each block fractionate together after partial acid or alkaline hydrolysis. Linkages between, or within, the segments are not known but, as a working hypothesis, and by analogy to known glycosphingolipids, it may be that several of the components are linked through inositol.

cleave ester bonds, and the isolation of ceramides from partial alkaline hydrolysates suggest that the fatty acids and phytosphingosine bases are in ceramides linked to the rest of the molecule through acid and alkali labile bonds perhaps involving the 1-hydroxyl group of the phytosphingosines. Inositol (0.25 μmol/μmol of total phosphorus, enzymatic assay) is equimolar with the ceramide and there is sufficient acid-hydrolyzable phosphate (0.38 μmol/μmol of total phosphorus) to support the hypothesis of ceramide linked through phosphate to inositol. This structure is supported by the preliminary identification of inositol phosphate among the products of partial acid hydrolysis and by the resistance of lipophosphonoglycan to alkaline phosphatase.

The stoichiometry of the compositional data (Ref. 5 and this paper) dictates that there should be about three such lipid groups for every galactosamine. The aminophosphonates may be involved in the linkage of the lipids, perhaps through the inositol moieties. In essence, then, one segment of both components of lipophosphonoglycan may be an oligomeric lipid containing three ceramide phosphate inositol moieties and six aminophosphonates. The galactosamine may also be linked to inositol, since the aminophosphonates, inositol, acid-hydrolyzable phosphate, and galactosamine are recovered together in fractions isolated from partial acid hydrolysates (Table IV).

The two components of lipophosphonoglycan differ with respect to their sugar constituents. The component with faster electrophoretic mobility contains glucose, mannose, and xylose, while the electrophoretically slower component of lipophosphonoglycan contains glucose, mannose, and galactose. Fractions C and B (Table II), which contain oligosaccharides of composition very similar to those shown in Fig. 5 (together with inositol, aminophosphonates, galactosamine, and acid-hydrolyzable phosphate), could be derived from the fast and slow components, respectively. These working models which bear a strong resemblance to, and are in part based on, the structure of the phytoglycolipids of plant seeds and leaves (22) are thus compatible with almost all of the analytical data. They do not account for the glucosamine or for all of the acid-hydrolyzable phosphate known to be present in lipophosphonoglycan nor, of course, for unknown constituents that may contribute to 20% of the mass of the molecule.

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