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The Isolation and Partial Characterization of Two Novel Sphingolipids from *Neurospora crassa*: Di(Inositolphosphoryl)ceramide and [(gal)$_3$glu]ceramide*

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SUMMARY

Neurospora crassa strains labeled uniformly with $^{32}P_i$ and [^{3}H]inositol exhibit at least six phospholipid components containing ^{3}H when separated by paper chromatography. One of the major components is phosphatidylinositol. Other components, which account for 40 to 60% of the lipid-extractable ^{3}H in various strains, are stable to mild alkaline methanolysis and appear to be sphingolipids with equivalent amounts of inositol and phosphorus. The major phosphosphingolipid was purified by means of differential solubility and by column chromatography on porous silica beads. This substance contains equivalent amounts of hydroxysphinganine and hydroxytetracosanoic acid and 2 eq each of myoinositol, phosphorus, and sodium. Alkaline degradation yielded 2 eq of inositol monophosphate and periodate degradation gave a C-15 fragment. The elemental composition of this compound also fits the formulation, (inositol-P)$_2$-ceramide.

A [^{3}H]inositol pulse-chase experiment carried out with an inositol-requiring mutant in exponential growth shows labeled inositol accumulating in the sphingolipid accompanied by decreased labeling in phosphatidylinositol and the acid-soluble fraction. These changes also occur when the chase is carried out during inositol starvation suggesting that degradation of phosphatidylinositol and formation of sphingolipid occurs in the absence of growth.

A neutral glycosphingolipid was also obtained as a by-product of the phospholipid purification. This substance is provisionally formulated as the ceramide tetrahexoside: [(gal)$_3$glu]-*N*-hydroxytetracosonyl-hydroxysphinganine.

Recent work has shown that a large fraction of the inositol-containing phospholipids in *Saccharomyces cerevisiae* are sphingolipids. These have one or two phosphoinositol groups and some also contain mannose (1, 2). A survey of other fungi for the presence of these compounds seemed worthwhile. We chose to first examine strains of *Neurospora crassa* for the presence of these inositol-containing sphingolipids, not only because of the general biochemical utility of this organism but especially because of the intriguing physiological and morphological effects that occur as a consequence of inositol starvation (3-8).

This paper reports on the presence in *N. crassa* of a major and novel inositol-containing phospholipid and a novel neutral glycosphingolipid.

EXPERIMENTAL PROCEDURES

Materials

Wild type *Neurospora crassa* strain RL21a was obtained from C. W. Slayman, Yale. Strain 89601a inos$^-$ and the wall-less slime mutant (FGSC 1118) was obtained from the Fungal Genetics Stock Center, Humboldt State College, Arcata, Calif. Strains 37401 inos$^-$ and 34486 chol$^-$ were obtained from the American Type Culture Collection. [2-^{3}H]Myoinositol and carrier-free $^{32}P_i$ were obtained from New England Nuclear Corp. Porous silica beads, Porasil, were obtained from Waters Associates, Framingham, Mass. Precoated silica gel thin layer plates were the product of Quantum Industries, Fairfield, N. J. *Escherichia coli* alkaline phosphatase was purchased from Worthington Biochemical Corp. Hydroxysphinganine was obtained from Cal Biochem.

Methods

Growth of Neurospora—Large scale growth of strain RL21a was carried out with forced aeration in Vogel's medium at 22° for 20 hours. Cells were harvested by filtration, washed with water, and extracted immediately. In some cases, the filtered cells were treated with cold 5% trichloroacetic acid for 1 hour, washed with water, and stored at −20°. Carbon source in all experiments was 2% sucrose. The slime mutant was maintained and grown in liquid culture at 30° as described (9).

Preparation of Sphingolipid Concentrate—Water was added to 1105 g (wet weight) of cells to make 8.2 liters and the mycelial suspension was blended briefly. To this mixture were added 1 volume of ethanol, ⅓ volume of diethyl ether, and 1/15 volume of pyridine. The mixture was adjusted to pH 8.5 with concentrated NH_4OH and refluxed with stirring at 54° for 2 hours. While warm, the mixture was filtered with suction through four layers of cheesecloth. The filtrate was adjusted to pH 5 with acetic acid and allowed to stand for a week at 5°. The bulk of the supernatant was siphoned off and discarded. The precipitate was suspended in the remaining supernatant and the mixture was centrifuged. The supernatant was discarded and the precipitate (7.66 mmoles of phosphorus) was suspended in 50 ml of pyridine-water (3:7, v/v).

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This suspension was added to a column (4 × 25 cm) of Li^+ form Chelex resin (Bio-Rad Laboratories) packed in water. Elution was carried out with pyridine-water (3:7, v/v) and all the phosphorus was eluted in 580 ml. The eluate was adjusted to pH 6.1 with acetic acid, a ½ volume of acetone was added, and the solution was stored at −20° for 48 hours. The precipitate was collected by centrifugation and the supernatant (1.9 mmoles of phosphorus) was discarded. The precipitate was washed twice with 100-ml portions of acetone and dried (5.9 g). The acetone washes (0.44 mmole of P) were discarded. The precipitate was suspended in 60 ml of water after which 192 ml each of CH_3OH and $CHCl_3$ were added. After warming briefly at 45° the suspension was centrifuged and the supernatant reserved. The pellet was extracted twice with 74-ml portions of $CHCl_3$-CH_3OH-H_2O (16:16:5). The combined solubles had 4.46 mmoles of phosphorus and the precipitate had 1.01 mmoles of phosphorus.

Surprisingly, no further phospholipid could be solubilized with the solvent mixture employed, although thin layer chromatography of the water suspended precipitate showed it to have a lipid spot at the R_F of Compound A (Fig. 1) which by its intensity (rhodamine) may have been roughly equal to the Compound A rendered soluble in the solvent, $CHCl_3$-CH_3OH-H_2O (16:16:5). There was also a very intense carbohydrate-positive (orcinol-H_2SO_4) spot at the origin of the thin layer chromatogram; no glycolipid above the origin was seen. No attempt was made to obtain lipid from this fraction since it appeared to be tightly complexed to unknown impurities.

To the combined soluble fraction (592 ml) was added 296 ml of CH_3OH and the mixture was stored overnight at −20°. The precipitate constituting the sphingolipid concentrate was collected by centrifugation at −20° and washed twice with 20-ml portions of acetone and dried in a stream of N_2. The dried product (0.27 mmole of phosphorus) weighed 0.45 g. This last step precipitated about one-half to two-thirds of the desired phospholipid product and virtually all of the glycolipid as judged by thin layer chromatography. This precipitate was seen to be virtually free of carbohydrate material that remained at the origin and relatively free of lipids with R_F values higher than the desired components; these less polar lipids remained in the supernatant fractions (4.13 mmoles of phosphorus). Further purification of the precipitate was achieved by column chromatography (Fig. 2) which showed that less than 30% of the total phosphorus in this concentrate eluted ahead of the desired lipids and virtually no phospholipid was eluted later.

Final Purification of Sphingolipids by Column Chromatography—To a column (2.5 × 200 cm) of NaOH-treated Porasil prepared exactly as previously described (2) was added the sample (0.18 mmole of phosphorus) dissolved in 30 ml of $CHCl_3$-CH_3OH-H_2O (16:16:5, v/v). This sample was composed of the final sphingolipid concentrate (110 μmoles of phosphorus) plus a glycolipid poor fraction (66 μmoles of phosphorus) which had been obtained from pilot columns run on the sphingolipid concentrate. Elution was carried out at 42° with the following schedule: 5.58 liters of $CHCl_3$-CH_3OH-H_2O (52:42:6, v/v), flow rate 8.74 ml per min; 2.48 liters of $CHCl_3$-CH_3OH-H_2O (51.7:40:8.3, v/v), flow rate, 2.81 ml per min; 1.8 liters of $CHCl_3$-CH_3OH-H_2O (16:16:5, v/v), flow rate 8.74 ml per min. Total phosphorus and hexose (mannose, standard) was determined on each fraction. These results plus those of thin layer chromatography led to pooling the fractions indicated in Fig. 2 (*(IP)$_2$C, Glycolipid*). These pooled fractions were taken to dryness *in vacuo*, dissolved in $CHCl_3$-CH_3OH-H_2O (16:16:5, v/v), and virtually quantitatively precipitated with 0.5 volume of CH_3OH at −20° overnight. The precipitates were taken to constant weight at 60° in a vacuum oven. For further analysis solutions of the phospholipid were made in water and of the glycolipid in $CHCl_3$-CH_3OH-H_2O (16:16:5, v/v).

Analytical Chromatography—Two-dimensional chromatography on silica gel-loaded paper was carried out as previously described (10). Silica gel thin layer chromatograms of intact lipids were developed with $CHCl_3$-CH_3OH-4.2 N NH_4OH (9:7:2, v/v) (Solvent I) or with $CHCl_3$-CH_3OH-acetic acid-H_2O (16:6:4:1.6, v/v) (Solvent II). ^{32}P-labeled lipids were located by exposure of the chromatogram to Kodak No-Screen x-ray film; spots were cut out and counted directly in a Triton-containing scintillation fluid (11).

Analytical Procedures—Unless otherwise noted, details of assay procedures were as previously described (2). These included: identification of long chain bases and fatty acid after HCl-methanolysis by thin layer chromatography and gas-liquid chromatography; quantitative assay of sodium, phosphorus, inositol, hexose, long chain base, fatty acids as esters after methanolysis, uronic acid, and hexosamine. Analyses of carbon, hydrogen, and nitrogen were carried out by Galbraith Laboratories, Inc. Carbohydrate assay after methanolysis of lipids and subsequent gas chromatographic assay of the trimethylsilyl ethers of methylglycosides was carried out according to the procedure of Desnick *et al.* (12) using mannitol as internal standard.

Analysis of Products of Alkaline Hydrolysis of Compound A—Compound A (3 μmoles of phosphorus) was treated for 15 hours at 37° in 1 ml of 1 N KOH. The solution was neutralized with 0.5 ml of 2 N formic acid and 1.5 ml each of $CHCl_3$ and CH_3OH were added. After mixing well the phases were separated and taken to dryness in a stream of N_2. The upper phase was redissolved in water and the lower phase was redissolved in $CHCl_3$-CH_3OH-H_2O (16:16:5, v/v). After aliquots were removed for the assay of free inositol, total phosphorus, and P_i, the aqueous phase was subjected to treatment with alkaline phosphatase. Treatment was carried out for 17 hours at room temperature in a mixture of 1 mg per ml of enzyme-0.02 M ammonium acetate, pH 8.6. Inositol was converted to the hexatrimethylsilyl derivative by taking the appropriate samples to dryness, adding 0.3 ml of silylating reagent, and heating for 48 hours at 60°. Appropriate blanks and standards were run simultaneously. The silylating reagent and gas-liquid chromatography of the trimethylsilyl ether was carried out as described previously (2).

Periodate Oxidation of Intact Lipids—A slight modification (2) of the method of Carter and Hirschberg (13) was employed to form long chain aldehydes by periodate oxidation of intact lipids and subsequently reduce these with borohydride to long chain alcohols which were identified and quantitated by gas-liquid chromatography of their trimethylsilyl ether derivatives. Hydroxysphinganine was run in parallel as a quantitative reference standard to which the detector responses of the unknowns were compared.

RESULTS

Characterization of ^{32}P and [^3H]Inositol-labeled Lipids Separated by Paper Chromatography—An inositol-requiring mutant of *N. crassa* was grown in the presence of $^{32}P_i$ and [2-^{3}H]inositol to ensure uniform labeling of the inositol-containing phospholipids. Extraction of lipids from cells in exponential growth phase was carried out by a procedure that was quite efficient (Fig. 1). Control extraction experiments with cells labeled only with [^{3}H]-inositol showed <1% of the counts remaining in the residue. The doubly labeled lipid extract after paper chromatography revealed six distinct ^{32}P spots (*A* to *F*, Fig. 1) that contained significant ^3H counts.

Spot E was judged to be phosphatidylinositol. It had the same R_F as yeast phosphatidylinositol in this system (Fig. 1) as well as on silica gel thin layer plates developed with Solvent I. Spot E was eluted from the paper (Fig. 1) and subjected to mild alkaline methanolysis (14), rendering all of the ^{32}P and ^3H counts water-soluble. The labeled material in the aqueous phase was identified as glycerophosphorylinositol by two-dimensional paper chromatography with the solvents previously described (15). This showed a single radioactive spot that was superimposed on the yeast glycerophosphorylinositol which was added as internal reference and detected chemically with periodate.

Spots A and *D* were eluted from the paper (Fig. 1) with $CHCl_3$-CH_3OH-H_2O (16:16:5, v/v) and subjected to mild alkaline methanolysis. No significant ^{32}P or ^3H counts from Spot D became water-soluble by the previously described (2) procedure, and all the radioactivity still migrated at the original R_F on silica gel thin layer plates (Solvent I). Because of the substantial water solubility of Spot A, the following alternate methanolysis procedure was employed. Equal volumes of the chromatogram eluate and 0.2 N NaOH in methanol were mixed

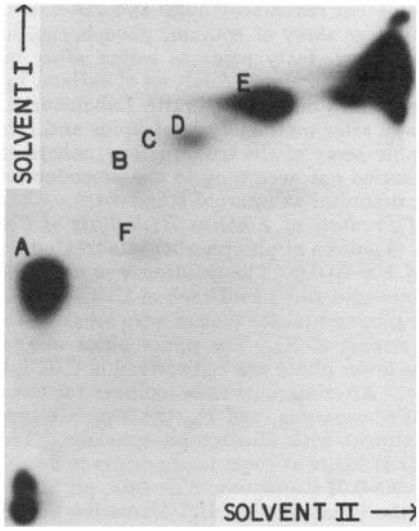


FIG. 1. Two-dimensional chromatography of a lipid extract prepared from cells uniformly labeled with $^{32}P_i$ and [^{3}H]inositol. *Neurospora crassa* (strain 89601a) was grown at 26° for 13.5 hours in Fries minimal medium supplemented with myoinositol (20.2 μg per ml) after inoculation with 0.5×10^6 conidia per ml. $^{32}P_i$ was added to a specific activity of 67.6 μCi per μmole and [2-^{3}H]-myoinositol added to a specific activity of 136 μCi per μmole. A 2-ml aliquot was filtered and washed with unlabeled medium and finally placed in cold 5% trichloroacetic acid. After 2 hours in the cold, the cells were washed twice with 10-ml portions of H_2O and stored at −20° overnight. The mycelial pellet was suspended in 2 ml of H_2O-ethanol-diethylether-pyridine (15:15:5:1, v/v) and treated at 60° for 15 min. After centrifuging, the supernatant was removed, and an additional 1 ml of extracting solvent was added to the residue and extraction was carried out at 60° for 15 min followed by centrifugation. The two supernatant extracts were combined and a 0.05-ml aliquot was subjected to two-dimensional chromatography (10) on silica gel-impregnated paper followed by autoradiography as described under "Experimental Procedures."

and incubated at 30° for 20 min followed by neutralization with 0.012 volume of glacial acetic acid. A control reaction mixture was instantly neutralized. The entire reaction mixture was subjected to two-dimensional silica gel thin layer chromatography using Solvent I followed by Solvent II. Autoradiography shows Spot A to be unaffected by this procedure. Thus, the absence of carboxylic ester bonds in Compounds A and D is indicated.

Table I shows the quantitative distribution of radioactivity on the chromatogram shown in Fig. 1. The data show that Spots A to D have the same ^{3}H:^{32}P ratio as Spot E, phosphatidylinositol, indicating strongly that Compounds A to D have equimolar amounts of inositol and phosphorus. Uniform ^{32}P labeling is assured because P_i is the sole source of P in the medium. Since the strain employed has a strict requirement for myoinositol, no endogenous synthesis can be anticipated. The only other factor which could complicate the interpretation of the count ratio as a mole ratio is if the [2-^{3}H]inositol might have been catabolized to other ^{3}H-containing substances. This catabolism appears unlikely since no ^{3}H counts were found in the non-inositol-containing phospholipids, phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine, which are clustered near the solvent fronts (Fig. 1) and which are reported in Table I as the sum of all other ^{32}P lipids.

Spot F appears to be a polyphosphoinositide because of its distinctly lower ^{3}H:^{32}P ratio; however, it has not been further investigated.

It was considered possible that the lipid pattern exhibited by strain 89601a (Fig. 1, Table I) was somehow related to the inability to synthesize inositol. Therefore, other strains were examined in a similar manner for their distribution of labeled inositol-containing phospholipids. The results shown in Table II indicate a basically similar distribution of [^{3}H]inositol in all the strains with the exception of the slime mutant which grows as a protoplast. In this mutant, equivalent amounts of Spots A and D were found. Phosphatidylinositol (E) and the unknown Compound A account for most of the lipid inositol in all these strains. Since Compound A was a major and unidentified lipid, we set out to isolate sufficient quantities of Compound A for further characterization.

Isolation of Compound A and Glycolipid—We initially assumed that Compound A (Fig. 2) was the substance with the composition, mannose (inositol-P)$_2$ceramide recognized in yeast (2) because of their similar R_F values on paper chromatography (9, 16). Therefore, we adopted a procedure for its isolation patterned after that employed in the preparation of yeast sphingolipids (2)

TABLE I

Radioactivity in lipids from Neurospora crassa (89601a) uniformly labeled with [^{3}H]inositol and $^{32}P_i$ and separated by two-dimensional paper chromatography

The lipid extract from doubly labeled cells was obtained and processed precisely as detailed in the legend to Fig. 1. ^{32}P-Labeled spots were located by autoradiography, cut out, and counted. The rest of the paper was cut up, counted, and reported as a sum. Each ^3H-containing spot was counted for 20 min.

Lipid spot(s)[a]	^{32}P	^{3}H	^{3}H:^{32}P
	cpm/zone		*E = 1.000*
A	9,732	1,992	1.01
B	91	15.9	0.86
C	72	14.1	0.96
D	532	101	0.93
E	7,402	1,505	1.00
F	112	8.0	0.35
Sum of all other ^{32}P lipids	59,940	0.0	0.00
Sum of all nondiscrete spots[b]	3,298	173	0.26

[a] Spots designated as in Fig. 1.

[b] All the rest of the paper was counted, however, the radioactivity is due primarily to streaking of major spots (Fig. 1).

TABLE II

Distribution of inositol-containing phospholipids in several strains of Neurospora crassa

Cells were grown in the presence of $^{32}P_i$ and [2-^{3}H]myoinositol, and lipid extracts were prepared and chromatographed essentially as indicated in the legend to Fig. 1. Choline assay medium (B460, Difco Laboratories) was used for strain 34486 supplemented with 8 μg per ml of choline and for strain 37401 supplemented with 1.3 μg per ml of myoinositol. Vogel's minimal medium was used for strain RL21a.

Strain	Inositol-containing lipids[a]						Sum (A to F)
	A	B	C	D	E	F	
	% ^{3}H-lipid						*% ^{32}P-lipid*
RL21a wild	38	0.4	0.4	1.3	60		15.8
34486 chol$^-$	57	1.3	0.9	3.2	37	0.1	15.9
37401 inos$^-$	60	1.2	0.6	4.5	34	0.3	26
89601 inos$^-$	55	0.4	0.4	2.8	41	0.2	23
Slime	24	0.4	1.6	21.4	53	0.3	17

[a] Lipid designations as in Fig. 1.

This procedure involving differential solubility produced a sphingolipid-rich concentrate ("Experimental Procedures") in which better than two-thirds of the phosphorus was in the desired compound and which we estimate was obtained in about 25% yield at this stage as judged by silica gel thin layer chromatography (Solvent I).

At this stage of purification it became evident that the *Neurospora* Compound A was not equivalent to the yeast compound. The *Neurospora* compound had a slightly higher R_F on thin layer chromatograms and it appeared to consist of two poorly resolved spots both of which were rhodamine-positive, but only the lower one giving a positive glycolipid test with the orcinol-H_2SO_4 reagent.

The sphingolipid concentrate was further purified on a column of porous silica beads (Porasil). It can be seen from Fig. 2 that

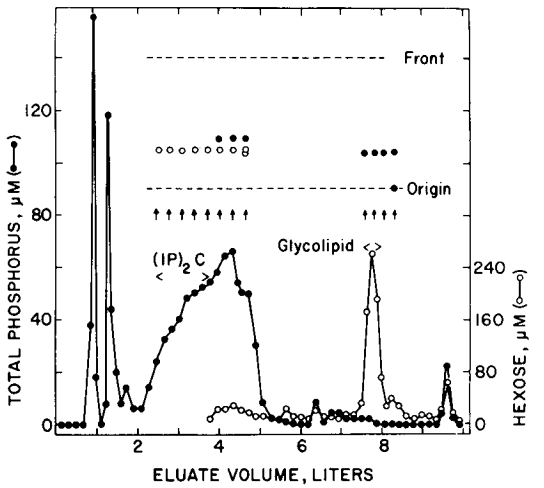


FIG. 2. Column chromatography of sphingolipid concentrate. Chromatography on Porasil was carried out as described under "Experimental Procedures." Total phosphorus (●——●) and hexose (○——○) was estimated on the fractions indicated. Silica gel thin layer chromatography was carried out on the fractions indicated (↑) and the results are shown schematically in the *inset*. Lipids were detected with a rhodamine spray (○) and glycolipids were detected with the orcinol-H_2SO_4 reagent (●). Fractions were pooled as indicated (<>). $(IP)_2C$, (inositol-P)$_2$-ceramide.

the majority of the phosphorus elutes in a band well before the major carbohydrate-containing material. In addition in this concentrate a glycolipid of lesser magnitude elutes in the tail end of the phospholipid peak. It can also be seen that the major glycolipid band is free of phosphorus. Based on thin layer chromatography, fractions containing Compound A ($(IP)_2C$, Fig. 2) and fractions containing the major glycolipid were pooled and obtained in a dry state for further analysis as indicated under "Experimental Procedures." We will refer hereafter to the major glycolipid as simply "glycolipid."

The final products each exhibited one spot when detected with rhodamine on silica gel chromatography with either Solvent I (R_F values: A, 0.30; glycolipid, 0.26) or Solvent II (R_F values: A, 0.085; glycolipid, 0.25).

Purified Compound A was mixed with a ^{32}P-labeled crude lipid extract and subjected to two-dimensional chromatography as in Fig. 1. The unlabeled Compound A was detected with rhodamine and corresponded with the ^{32}P zone designated A, detected by autoradiography. When similar comparisons were attempted by thin layer chromatography (Solvent I) it was noted that ^{32}P-labeled Spot A eluted from paper chromatograms gave two very close spots on thin layer chromatograms in about a 10:1 ratio. The isolated Compound A corresponded exactly to the upper major ^{32}P-labeled spot in experiments where both were mixed prior to thin layer chromatography (Solvent I).

Chemical Characterization of Compound A—Analysis showed purified Compound A could be formulated as the disodium salt of a sphingolipid composed of 1 eq of ceramide and 2 eq of phosphoinositol (Table III). Tests for hexosamine and uronic acid were negative.

Thin layer chromatography of the long chain base fraction after acid methanolysis showed it to be composed solely of ninhydrin-positive material at the R_F of hydroxysphinganine (phytosphingosine) with a trace of high R_F material which was presumably anhydrophytosphingosine, a well known artifact of hydrolysis. Gas-liquid chromatography of the long chain base fraction (Table IV) confirmed these observations and showed that the C-18 base predominated with a trace of the C-20 base evident. Retention times expected for the C-20 base were extrapolated from the data of Carter and Gaver (17).

Thin layer chromatography of the fatty acid methyl ester frac-

TABLE III

Composition of purified sphingolipids

Lipid	C	H	N	P	Na	Long chain base	Fatty acid	Inositol	Hexose
	% by weight					*moles/g anhydrous equivalent weight*			
A									
Experimental	51.9	8.63	1.22	4.88	3.40	0.90	0.75	2.14	≤.05
Calculated, anhydrous[a]	53.5	8.73	1.16	5.11	3.79	1.00	1.00	2.00	0
Calculated, plus $2H_2O$	51.9	8.80	1.12	4.96	3.68				
Glycolipid									
Experimental	56.3	9.20	0.91	≤0.06	≤0.19	1.09	0.91	0	3.91[c], 3.43[d]
Calculated, anhydrous[b]	59.5	9.39	1.05	0	0	1.00	1.00	0	4.00
Calculated, plus $4H_2O$	56.4	9.53	1.00	0	0				

[a] Based on the sodium salt of a compound containing two phosphoinositol moieties and a ceramide composed of hydroxysphinganine and hydroxytetracosanoic acid, $C_{54}H_{105}O_{21}NP_2Na_2$, equivalent weight, 1212.4.

[b] Based on a compound containing four hexose moieties and a ceramide composed of hydroxysphinganine and hydroxytetracosanoic acid, $C_{66}H_{125}O_{25}N$, equivalent weight, 1332.7.

[c] Hexose assay by phenol-H_2SO_4 method with three parts galactose plus one part glucose as standard.

[d] Sum of galactose and glucose estimated by gas-liquid chromatography.

TABLE IV

Gas chromatographic identification of major long chain bases obtained by methanolysis of purified sphingolipids

Lipid	Peak	Tentative designation	Total detector response	Retention time	
				Found	Expected[a]
			%	*min*	
Compound A	1		11	4.1	
	2	Hydroxysphinganine	82	8.1	8.1
	3	Hydroxyeicosasphinganine	7	15.1	15.1
Glycolipid	1		4	4.1	
	2	Hydroxysphinganine	91	8.1	8.1
	3	Hydroxyeicosasphinganine	5	15.1	15.1

[a] Values were obtained from sphinganine and hydroxysphinganine standards and from the data of Carter and Gaver (17).

TABLE V

Gas chromatographic identification of major fatty acid esters obtained by methanolysis of purified sphingolipids

Lipid	Peak	Identity	Total detector response	Retention time	
				Found	Expected[a]
			%	*min*	
Compound A	1		3	6	
	2	OH 22:0	4	8.6	8.6
	3	24:0	1	9.8	9.8
	4		2	12.1	
	5	OH 24:0	86	16.8	16.8
	6		4	23.8	
Glycolipid	1		2	6	
	2	OH 22:0	3	8.6	8.6
	3	24:0	1	9.8	9.8
	4		5	12.1	
	5	OH 24:0	88	16.8	16.8
	6		1	23.8	

[a] According to data obtained from standards.

tion after HCl-methanolysis showed it to be exclusively composed of material at the R_F expected for methyl esters of monohydroxy fatty acids. Gas-liquid chromatography of the silylated fatty acid ester fraction confirmed these observations and showed that the major component had the retention time found for the 2-hydroxy C-24 acid derivative (Table V).

Elemental analysis (Table III) is consistent with the formulation of Compound A as (inositol phosphate)$_2$Na$_2$ceramide, the ceramide being composed of hydroxytetracosanoic acid and hydroxysphinganine. Treatment with aqueous 1 N KOH at 37° for 15 hours resulted in almost all the phosphorus being converted to a water-soluble organic form. Treatment of this product with phosphatase yielded equivalent amounts of free inositol and P_i (Table VI). We conclude, therefore, that the product of alkaline hydrolysis is inositol monophosphate(s) accounting for all of the phosphorus and inositol present in Compound A.

Although we have no direct evidence that the fatty acid is in an amide linkage, we can rule out the presence of an ester since Compound A survives the mild alkaline methanolysis procedure that completely deacylates ester-containing lipids.

TABLE VI

Analysis of water-soluble products of alkaline hydrolysis of Compound A

Compound A (3.0 μmoles of phosphorus) was treated with 1 N aqueous KOH for 15 hours at 37° and processed as described under "Experimental Procedures."

	Water-soluble fraction	
	No treatment	After phosphatase
	μmoles	
Total phosphorus	2.5	2.5
Inorganic phosphate	0.01	2.4
Free inositol	0.01	2.4

Treatment of Compound A with periodate followed by reduction with borohydride gave a 64% yield of pentadecanol and heptadecanol in the expected ratio (Table IV) showing that the 3- and 4-hydroxyl groups of the long chain bases were unsubstituted.

The facile formation of inositol monophosphate by alkaline hydrolysis suggests that phosphodiesterbonds are the likely bonds linking the inositols to the lipid moiety of Compound A and possibly to each other. The alkaline lability of such bonds is probably due to the intermediate formation of cyclic inositol phosphate. Inositol phosphorylceramide from yeast exhibits a similar alkaline lability (2). It remains to be established which positions in both inositols participate in the phosphodiester bonds. Since the 3- and 4-hydroxyls of the long chain base appear to be unsubstituted, the 1-hydroxyl of the long chain base and the hydroxyl of the fatty acid remain as possible sites for the attachment of the phosphoinositol group(s). If Compound A is like all other complex sphingolipids, one should anticipate substitution at the C-1 hydroxyl of the long chain base.

Chemical Characterization of Purified Glycolipid—Carbohydrate assay was carried out after treating the lipid with anhydrous 1 N HCl in methanol at 80° for 24 hours followed by gas-liquid chromatography of the trimethylsilyl methylglycosides (12). The only sugars observed were galactose (1.93 μmoles per mg) and glucose (0.65 μmole per mg). Direct chemical analysis of total hexose by the phenol-H_2SO_4 method gives a significantly higher value, 2.93 μmoles per mg (Table III). A sample was also hydrolyzed with 2 N H_2SO_4 for 5 hours at 100°. After extraction with petroleum ether the aqueous phase was neutralized with Dowex 1-HCO_3^- and chromatographed on cellulose thin layer with butanol-pyridine-H_2O (6:4:3, v/v). Only spots at the R_F values of glucose and galactose were observed after detection with the *p*-anisidinephthalate reagent (18).

A test for hexosamine was negative and a carbazole (19) assay for uronic acid gave a value of 0.3 μmole per mg. Hexoses react in the carbazole test to some extent and the galactose and glucose present could be expected to account for this low apparent value for uronic acid.

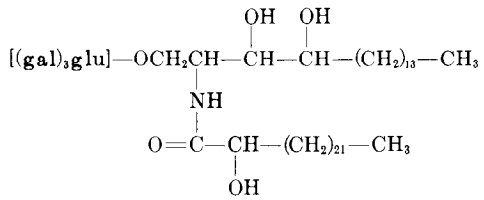
Equivalent amounts of long chain base (20) and fatty acid were found, indicating that the glycolipid was a sphingolipid (Table III). Examination of the appropriate fractions after HCl-methanolysis by thin layer chromatography and gas-liquid chromatography gave the same results as found with Compound A, namely, the principal lipid components appeared to be 2-hydroxytetracosanoic acid and hydroxysphinganine (Tables IV and V).

The glycolipid (1 μmole of hexose) was treated for 20 min at 30° in 2 ml of 0.1 N KOH in $CHCl_3$-CH_3OH-H_2O (16:53:5). After neutralization with 0.2 ml of 1 N acetic acid, 1 ml of $CHCl_3$

and 1.15 ml of H_2O were added. All of the original carbohydrate was recovered in the organic phase. Silica gel thin layer chromatography (Solvent I) showed only the original rhodamine-positive, orcinol-H_2SO_4-positive spot. This stability toward mild alkaline methanolysis indicates absence of acyl ester groups. We conclude therefore that the fatty acid is amide linked.

Periodate oxidation of the intact lipid followed by borohydride reduction gave 96% of the expected yield of pentadecanol plus heptadecanol and in the expected ratio (Table IV). Therefore, the 3- and 4-hydroxyl groups of the long chain bases are unsubstituted.

The sugar and nitrogen values obtained (Table III) are slightly lower than anticipated for a ceramidetetrahexoside and would also fit a ceramidepentahexoside. However, the gas-liquid chromatography results show a galactose to glucose ratio of exactly 3:1, and total hexose estimated chemically (Table III) shows a value reasonably close to 4 moles/1332 g. Since there is no objective evidence for any other sugar, or any other component for that matter, we would conclude that all data best fit a provisional formulation as a ceramidetetrahexoside:



The glycolipid isolated was a fortuitous by-product in the isolation of Compound A and so it is difficult to make a judgement about how much of this substance is present. We have, however, subjected crude lipid extracts from strains 89601a and 37401 to two-dimensional silica gel thin layer chromatography (Solvents I and II) and sprayed the plates with the orcinol-H_2SO_4 reagent. Many spots are evident with the spot in the region of the isolated glycolipid being the most polar and the second or third most intense.

Turnover of Major Inositol-containing Lipids—When growing yeast cells, uniformly labeled with [^{3}H]inositol, were transferred to unlabeled growth medium, phosphatidylinositol lost label, about half of which accumulated in the major yeast sphingolipid, mannose (inositol-P)ceramide (11). We have carried out a similar experiment with an inositol-requiring strain of *N. crassa*. The chase portion of the experiment was carried out in complete growth medium as well as in an inositol-deficient medium (Table VII). The results show that in a 5-hour chase, substantial decreases in label are seen in the acid-soluble fraction and in phosphatidylinositol. These decreases are accompanied by increased labeling of sphingolipid and the culture medium. These changes occur even when growth is limited by inositol deficiency.

TABLE VII

Turnover of major inositol-containing pools in [^{3}H]inositol pulse-chase experiment

Strain 37401 inos$^-$ was grown for 14 hours in Fries medium supplemented with myoinositol (20 μg per ml) after inoculation with 0.5×10^6 conidia per ml. $^{32}P_i$ was added to a final specific activity of 30 μCi per μmole and [2-^{3}H]myoinositol to a specific activity of 136 μCi per μmole. Aliquots (2 ml) were filtered on a Millipore filter and washed rapidly with unlabeled medium; those samples destined to be incubated without inositol were washed with inositol-free medium. These washed samples were then incubated in 12 ml of fresh unlabeled complete medium, with and without 20 μg per ml of myoinositol. After 5 hours the medium was removed by filtration and the mycelia was treated with trichloroacetic acid, washed with water and lipid extraction, and chromatography performed as indicated in the legend to Fig. 1. The ^{3}H in each fraction has been calculated on the basis of volume of chase medium.

Component	^{3}H distribution		
	Zero time	Chase plus inositol	Chase minus inositol
	cpm/ml medium		
Phosphatidylinositol	5,425	3,925	3,235
Di(inositolphosphoryl)ceramide	9,575	17,663	16,067
Spot D[a]	717	913	1,716
Acid-soluble[b]	8,899	4,026	2,776
Culture medium	479	2,033	1,245

[a] Designation as in Fig. 1.

[b] ^{3}H in trichloroacetic acid-soluble fraction plus subsequent H_2O wash.

DISCUSSION

Although Compound A is a novel substance, the composition, Na_2(inositol-P)$_2$-ceramide clearly relates it to the yeast inositol phosphorylceramides that contain one or two phosphoinositol moieties (1, 2). It is also related to the more complex plant lipid, phytoglycolipid, extensively investigated by Carter and co-workers (21). This relationship is underlined by the fact that in all these substances are found very long chain (22 to 26 C) hydroxy fatty acids and trihydroxy long chain bases.

Spots B, C, and *D* (Fig. 1) have chromatographic mobilities similar to the monophosphoinositol-containing sphingolipids we have characterized in yeast (2, 16), and since the labeling patterns we have reported here demonstrate a 1:1 ratio of phosphorus and inositol, it seems likely that this group of compounds in *Neurospora* are monophosphoinositol sphingolipids.

Several interesting consequences of inositol starvation in *N. crassa* have been noted. The lethal effects of inositol starvation observed with inositol-requiring mutants (3) termed "inositol-less death" serves as the basis of a widely used technique of mutant selection (4). It was proposed that autolysis and death ensue when proteases are released from particles enclosed by an inositol lipid-rich membrane that ruptures when cells are starved of inositol (6). Striking morphological changes occur in inositol-starved cells (5). It has also been shown that sugar transport by cultures of an inositol-requiring mutant is rapidly and severely affected by changing the inositol content of the medium (7). The continued breakdown of phosphatidylinositol and synthesis of (inositol-P)$_2$ceramide which occurs even when growth is limited by inositol starvation (Table VII) may indicate that the deleterious effects of inositol starvation could not only be due to decreased cellular levels of phosphatidylinositol but also due to aberrantly high levels of the inositol-containing sphingolipids.

The recognition of a hitherto unknown array of inositol-containing lipids in *Neurospora* and the availability of methods for their study should provide an opportunity for the study of their role in membrane structure and function. It seems worthwhile to examine further the details of metabolism of the inositol-containing phospholipids as related to the various phenomena noted above. Pulse-chase experiments in yeast suggested that phosphatidylinositol can apparently serve as a phosphoinositol precursor of the major yeast phosphosphingolipid, mannose (inositol-P)$_2$ceramide (11). The results of a similar experiment carried out with *Neurospora* (Table VII) are not inconsistent with these

yeast findings; however, the very large labeled acid-soluble pool present at the beginning of the chase precludes an unambiguous interpretation. A large part of the label that accumulates in the sphingolipid must come from the acid-soluble pool either directly or via phosphatidylinositol as intermediate.

Complex neutral and acidic glycosphingolipids are well known in animal tissues (22). Although monoglycosylceramides have been widely observed in plants and fungi (22, 23), to our knowledge ceramideoligohexosides as complex as the glycolipid isolated have not yet been described from these sources.

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