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Inositol Phosphorylceramide, a Novel Substance and the Chief Member of a Major Group of Yeast Sphingolipids Containing a Single Inositol Phosphate*

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SUMMARY

About one-third of the nondeacylatable phospholipids in *Saccharomyces cerevisiae* have been shown to be a group of sphingolipids with a single phosphoinositol moiety. This group of lipids was obtained richly concentrated in high yield by several simple steps involving differential solubility. Four components of this group were further purified by liquid chromatography on base-treated porous silica beads. The major component of this group proved to be the novel substance, inositol phosphorylceramide, the ceramide consisting of hydroxysphinganine and a hydroxy C-26 fatty acid. Two other inositol phosphorylceramides were purified which differed from the major component in their ceramide moieties. Two glycolipids are minor members of this group of mono-inositol-containing sphingolipids and one of these was purified, and had a composition consistent with its formulation as a mannosylinositol phosphorylceramide.

The major inositol phosphorylceramide could be cleaved quantitatively by alkali to give an inositol monophosphate and could be cleaved by periodate to give a C-15 fragment. Periodate treatment followed by borohydride reduction and HCl hydrolysis gave a significant yield of phosphoserinol. These facts suggest strongly that the phosphoinositol moiety is attached to position 1 of the hydroxysphinganine.

Data obtained with cells uniformly labeled with [^{3}H]-inositol suggest that all significant (>1% lipid ^{3}H) inositol-containing lipids in yeast have now been chemically characterized.

The phospholipids of *Saccharomyces cerevisiae* have received renewed attention since research in membrane biochemistry has focused on this eukaryotic organism which can be conveniently manipulated by genetic and nutritional means. The inositol which is found in about one-third of the phospholipid of *S. cerevisiae* represents about 90% of the total cellular inositol content (1).

Only some of the inositol-containing lipids have been well characterized. About 60% of the inositol-containing lipid P is phosphatidylinositol with approximately 1% found as diphosphoinositide and triphosphoinositide (2–4). In contrast to these phosphoglycerides, well known constituents of animal membranes, a distinguishing feature of the balance of the inositol phospholipids of *S. cerevisiae* is their stability to deacylation by mild alkaline methanolysis (5). Two-dimensional chromatograms of the nondeacylatable fraction of lipids from cells uniformly labeled with $^{32}P_i$ and [^{3}H]inositol or [^{14}C]inositol show four radioactive phospholipid spots, designated "2," "5," "6," and "7" (5). Each of these spots had radioactive isotope ratios equal to that of phosphatidylinositol, indicating an equimolar phosphorus and inositol content. Spot 2 was the principal component of the nondeacylatable fraction and was judged to be most polar by virtue of its lowest R_F on two-dimensional chromatograms on silica gel impregnated paper. This substance was isolated and found to be a novel sphingolipid with the composition mannose (inositol-P)$_2$ ceramide (6). The only information concerning the identity of Spots 5, 6, and 7 was the observation that [^{14}C]deoxyglucose was rapidly incorporated into two lipids with R_F values similar to that of these less polar nondeacylatable lipids, thus indicating that perhaps two of these unknown inositol-containing phospholipids might be glycolipids (5).

The purpose of the research reported in this paper is to clarify the chemical nature of these unidentified inositol-containing phospholipids. This work has disclosed the novel sphingolipid, inositol phosphorylceramide, and establishes the novel natural occurrence of mannosylinositol phosphorylceramide.

EXPERIMENTAL PROCEDURES

Growth of Yeast for Large Scale Lipid Isolation—A strain of *S. cerevisiae* was grown on a complex medium with glucose as carbon source (3) to an absorbance (650 nm) of approximately 22. Growth was terminated by the addition of trichloroacetic acid to obtain a final concentration of 5%. After standing 15 min, the cells were washed twice with 0.5% (w/v) KH_2PO_4 and once with 0.015 M potassium phosphate buffer, pH 7.4. The cells, suspended in the buffer, were heated for 7 min at 95° and frozen for storage. Prior to extraction the thawed cells (approximately 240 g dry

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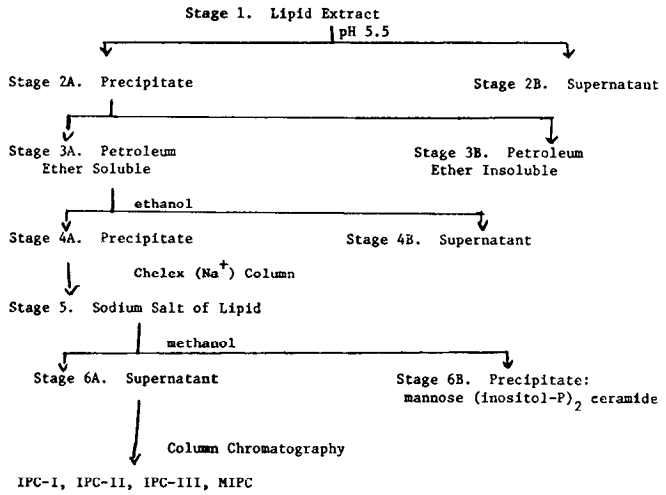


FIG. 1. Outline of sphingolipid isolation. Details are given under "Experimental Procedures."

weight) were washed with water and resuspended in enough water to bring the volume to 3336 ml.

Preparation of Sphingolipid Concentrate—Fig. 1 outlines the scheme employed to prepare a sphingolipid concentrate. To 1 volume of cell suspension, 1 volume of 95% ethanol, ⅓ volume of diethyl ether, and 1/15 volume of pyridine (1) were added and the mixture warmed at 57° for 30 min with frequent agitation. Cells were then removed immediately without cooling by centrifugation at room temperature and the lipid extract decanted. The extract (Stage 1) was adjusted to pH 5.5 with glacial acetic acid and allowed to stand at 0° for 48 hours. After centrifugation the supernatant was discarded. The precipitate (Stage 2A) was suspended in 600 ml of petroleum ether (37.2°–49.6°) and centrifuged at room temperature. Insoluble non-lipid material was discarded and an equal volume of absolute ethanol was added to the soluble supernatant (Stage 3A) which was allowed to stand at 0° overnight. After centrifuging at 0°, the precipitate (Stage 4A), composed almost entirely of lipids chromatographically more polar than phosphatidylinositol, was redissolved in 164 ml of Solvent A, $CHCl_3$-CH_3OH-H_2O (16:16:5, v/v). The lipid was converted to the sodium form by applying it to a Chelex 100 (Na^+, 100 to 200 mesh, Bio-Rad Laboratories) column packed in Solvent A. This resin had been prepared in a manner similar to that described by Carter and Weber (7). After washing with water to remove fines, a slurry of the resin was adjusted to pH 8.0 with 2 N H_3CCOOH. The resin was washed with CH_3OH and Solvent A and packed into a column. A column of 850 ml of resin was used for 2.5 mmoles of lipid phosphorus. The column was rinsed with Solvent A made basic by adding 0.47 ml of 0.05 N NaOH to 1 liter. After the sample was applied, approximately 1 column volume of the basic solvent was used to elute the sample (Stage 5). If the elution of the column was carried out at a slightly acidic pH, mannosyldiinositol diphosphorylceramide was completely retained along with some of the monoinositol phosphorylsphingolipids.

Mannosyldiinositol diphosphorylceramide was largely eliminated from the Chelex eluate (Stage 5) by precipitation with 0.5 volume of CH_3OH at room temperature. The supernatant (Stage 6A) was then dried *in vacuo* and redissolved in Solvent A for application to a Porasil column.

Preparation of Porasil Columns—Porasil A (60) (75–125 μm, Waters Associates, Inc.) was washed successively with Solvent A, $CHCl_3$-CH_3OH (1:1, v/v), and CH_3OH. After most of the solvent was removed by suction on a sintered glass funnel overnight, the Porasil was dried to constant weight in a 45° oven. Porasil (223 g) was added with stirring to 3.12 liters of 0.05 N NaOH in CH_3OH-H_2O (97.5:2.5, v/v) and stirred 8 min. After settling by gravity, the Porasil was washed five times with 3.12-liter portions of CH_3OH-H_2O (1:1, v/v), two times with 3.12-liter portions of CH_3OH, and two times with 3.12-liter portions of $CHCl_3$-CH_3OH (1:1). All columns were packed with the base-treated adsorbant in $CHCl_3$-CH_3OH (1:1, v/v) and then equilibrated with $CHCl_3$. After use these columns could be recycled *in situ* by washing successively with Solvent A, $CHCl_3$-CH_3OH (1:1, v/v), and $CHCl_3$.

Initial Column Chromatography of Sphingolipid Concentrate, Stage 6A—A typical initial separation of Stage 6A lipid was carried out as follows. To a Porasil column (2 × 94 cm) were added 600 μmoles of lipid phosphorus dissolved in 50 ml of $CHCl_3$-CH_3OH-H_2O (65:25:1, v/v). ^{32}P-labeled standards were added as indicated in the legend to Fig. 2. The column was eluted with 4.47 liters of $CHCl_3$-CH_3OH-H_2O (65:35:2.5, v/v), 0.91 liter of $CHCl_3$-CH_3OH-H_2O (65:55:8, v/v), and 0.98 liter of Solvent A. The flow rate was 8.8 ml per min. Total phosphorus determinations were made on 1-ml aliquots from each 69.9-ml fraction. A Packard Tri-Carb scintillation counter was used to measure Čerenkov radiation from 20-ml aliquots of each fraction (Fig. 2, *upper panel*). Key fractions were chromatographed on silica gel plates (Quantum Industries) developed with $CHCl_3$-CH_3OH-4.2 N NH_4OH (9:7:2, v/v) (8). Rhodamine was used to detect lipids and an overspray of orcinol-H_2SO_4 (9) indicated which were glycolipids. The results of thin layer chromatography and the elution patterns of ^{32}P-labeled reference compounds and total phosphorus were all guides in pooling the fractions containing the compounds of interest as shown in the *upper panel* of Fig. 2.

The pooled samples were subjected to column chromatography as above, again pooling appropriate fractions by the criteria indicated above. At this point it was recognized that insufficient resolution was achieved; therefore, chromatography was carried out with longer columns at an elevated temperature. Prior to final chromatography, pooled column fractions were taken to dryness *in vacuo* at approximately 40°. After dissolving in Solvent A, the sphingolipids were precipitated after addition of 0.5 volume of methanol and standing at 0° for several hours; this procedure was helpful in removing solvent impurities.

Final Purification of Inositol Phosphorylceramides I and II—A portion of the lipids which had been twice chromatographed and isolated on the basis of containing no carbohydrate was subjected to further chromatography. This fraction gave a N/P ratio of 0.97 (Table II, Fraction IPC-I + IPC-II[1]). A 55-mg sample of this fraction dissolved in 15 ml of Solvent A was applied to a Porasil column (200 × 2.5 cm) fitted with a water jacket to maintain a temperature of 42°. The column was eluted with 6.12 liters of $CHCl_3$-CH_3OH-H_2O (65:37:2.7, v/v) followed by 1.44 liters of Solvent A at a flow rate of 8.6 ml per min. One-milliliter aliquots from each 60-ml fraction were used for total phosphorus determination. The results are shown in Fig. 2, *middle panel*, and the fractions indicated were pooled and taken to dryness *in vacuo*. They were redissolved in Solvent A, centrifuged, and precipitated at 0° by addition of 0.5 volume of methanol. The final samples were dried at 120° for 2 hours and stored in a desiccator at room temperature. No evidence of breakdown was observed after several months

Final Purification of Inositol Phosphorylceramide III and Mannosylphosphorylceramide—The twice chromatographed fractions that were pooled on the basis of the presence of orcinol-positive material on thin layer chromatograms still contained non-glycolipid as well. A portion of this fraction, 44 μmoles of phosphorus in 28 ml of Solvent A, was applied to a Porasil column (2.5 × 200 cm) and eluted with 5.58 liters of $CHCl_3$-CH_3OH-H_2O (65:40:3, v/v) at a flow rate of 8.7 ml per min and at a temperature of 42°. Aliquots of the 61.9-ml fractions were assayed for total phosphorus with the results shown in the *lower panel* of Fig. 2. Thin layer chromatography was carried out on appropriate fractions and lipids and glycolipids were detected as indicated above. The peak eluted at about 2.5 liters (Fig. 2, *lower panel*) contained a single non-glycolipid spot. The first part of the next broad peak contained a single non-glycolipid spot of lower R_F, whereas the last part of this peak contained a single glycolipid spot. The bulk of the peak was a mixture of the glyco- and non-glycolipid. Fractions from each side of this peak were pooled as indicated in Fig. 2, *lower panel* (*IPC-III, MIPC*). These were evaporated to dryness *in vacuo*, redissolved in Solvent A, centrifuged, and precipitated at 0° after addition of 0.5 volume of methanol. The precipitates were redissolved in Solvent A and used as such for further analysis. Insufficient material was available to prepare enough dried samples for accurate handling.

Preparation of ^{32}P-labeled Lipid with High Specific Activity—A culture of cells was grown in the same manner as above except with

[1] The abbreviations used are: IPC, inositol phosphorylceramide. MIPC, mannosylinositol phosphorylceramide.

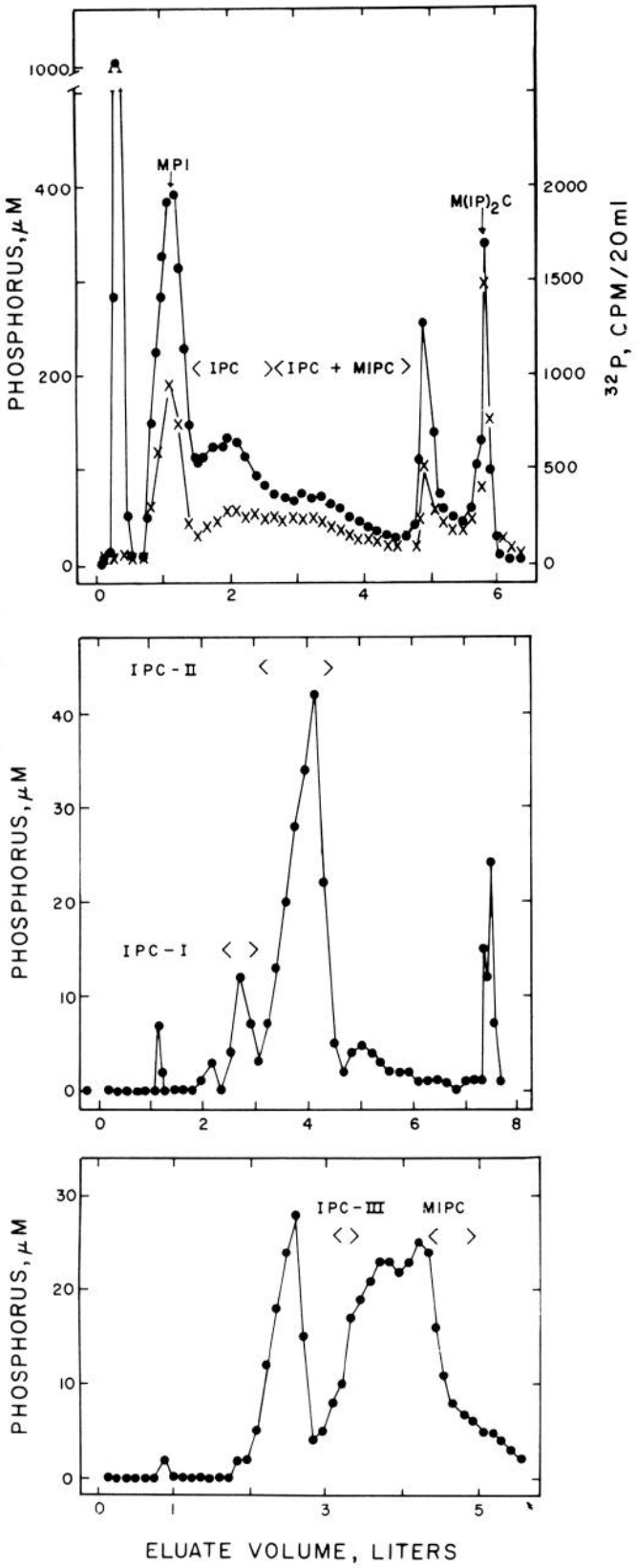


FIG. 2. Purification of inositolphosphorylceramides and mannosylinositolphosphorylceramide by column chromatography on Porasil. *Upper panel*, the sample consisting of lipid (*Stage 6A*, Fig. 1) was chromatographed at room temperature on a column (2 × 94 cm) as described under "Experimental Procedures." Included as reference markers were [^{32}P]phosphatidylinositol (*MPI*, 52,000 cpm); [^{32}P]inositol phosphorylceramide, primarily species II (*IPC*, 26,000 cpm); [^{32}P]mannosylinositol phosphorylceramide; (*MIPC*, 15,000 cpm); and [^{32}P]mannosyldiinositol diphosphoryl-

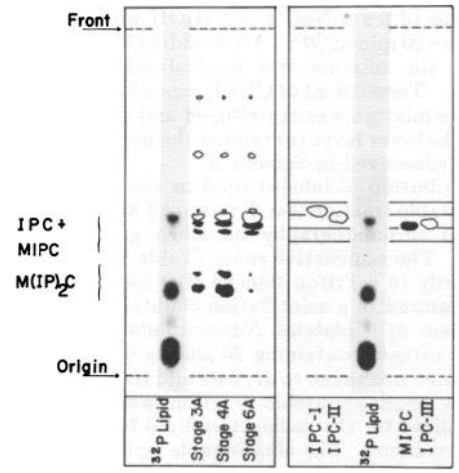


FIG. 3. Thin layer chromatography of various stages in the isolation of the sphingolipids. Silica gel plates were developed with $CHCl_3$-CH_3OH-4.2 N NH_4OH (9:7:2, v/v). The nondeacylatable fraction of a total lipid extract from cells uniformly labeled with ^{32}P and [2-^{3}H]inositol was added to each lane of the chromatograms. Radioactivity was detected by autoradiography and the original photograph is reproduced. Lipids detected by rhodamine are shown schematically with the spot sizes indicating relative intensities and their location relative to the internal ^{32}P lipid extract. Glycolipids, detected with orcinol-H_2SO_4, are indicated as *filled spots*. The *left panel* shows various stages of purification described under "Experimental Procedures." Approximately 60 nmoles of phosphorus per lane were chromatographed. The *right panel* compares the chromatography of the crude ^{32}P-labeled nondeacylatable fraction with each of the final purified sphingolipids (about 5 nmoles of phosphorus per lane). Abbreviations are: *IPC-I, -II, -III*, inositol phosphorylceramides; *MIPC*, mannosylinositol phosphorylceramide; *M(IP)$_2$C*, mannosyldiinositol diphosphorylceramide.

no added KH_2PO_4 and 6 mCi of carrier-free $H_3{}^{32}PO_4$ (New England Nuclear Corp.) were added to 5 ml of the medium. Lipids were extracted with 1.5 ml of solvent as described above and again with 0.5 ml of solvent.

Lipids were first separated on silica gel-impregnated. EDTA-treated paper as described earlier (5). Relevant spots detected by autoradiography were eluted with Solvent A. These were further purified once or sometimes twice by silica thin layer chromatography in one dimension with the solvent, $CHCl_3$-CH_3OH-4.2 N NH_4OH (9:7:2, v/v). The [^{32}P]inositol phosphorylceramide sample used in Fig. 3 was purified only on paper.

Measurement of Distribution of Labeled Inositol and Phosphorus Containing Lipids in Crude Lipid Extract—A 10-ml culture was grown on complex medium with 4% (w/v) glucose (3) with 300 μCi of [2-^{3}H]myoinositol (3.09 Ci per mmole, New England Nuclear Corp.) and 3 mCi of carrier-free $H_3{}^{32}PO_4$. The lipid was extracted as above after growth was terminated with 5% trichloroacetic acid at an absorbance (650 nm) of 3.2.

A nondeacylatable lipid fraction was prepared from this doubly labeled crude lipid extract as follows. Two-thirds of the extract was taken to dryness and redissolved in 0.5 ml of Solvent A. An

ceramide (*M(IP)$_2$C*, 12,815 cpm). Each eluate fraction was assayed for total phosphorus and 20 ml of each fraction were assayed for ^{32}P by Čerenkov radiation. Phosphorus recovery was 95%. The fractions that were pooled for further purification are indicated: < >. *Middle panel*, final chromatographic purification of inositol phosphorylceramides I and II. The partially purified sample was chromatographed on a column (2.5 × 200 cm) at 42° as described under "Experimental Procedures." Total phosphorus was assayed and fractions were pooled as indicated. *Lower panel*, final chromatographic purification of inositol phosphorylceramide III and mannosylinositol phosphorylceramide. The partially purified sample was chromatographed on a column (2.5 × 200 at 42° as described under "Experimental Procedures." Total phosphorus was determined for each fraction and eluates were pooled as indicated based on thin layer chromatography of key fractions.

equal volume of 0.2 N NaOH in CH_3OH was added and the tube incubated for 20 min at 30°. After adding 0.57 ml of 0.5% aqueous Na_2EDTA, the mixture was neutralized with 0.1 ml of 1 N H_3CCOOH. Then 0.5 ml of $CHCl_3$ was added, and after vigorous shaking, the mixture was centrifuged and the upper layer was removed. The lower layer containing the nondeacylatable lipid was dried and redissolved in Solvent A.

The distribution of labeled lipid in the crude extract and the nondeacylatable fraction was determined after separation by two-dimensional chromatography on silica gel paper as described earlier (4). The radioactive zones (Table VIII) were cut out and added directly to a Triton-toluene-H_2O containing counting fluid (1) and measured in a scintillation counter.

Preparation of Unlabeled Nondeacylatable Lipid Fraction—A crude lipid extract containing 20 μmoles of phosphorus and prepared as above was taken to dryness and redissolved in 3 ml of Solvent A. A nondeacylatable fraction was prepared exactly as described above for the radioactive lipid but with a proportional increase in volume. The alkali-stable lipids were assayed as described in Table IX.

Gas Chromatography—A Packard Series 7500 gas chromatograph fitted with a hydrogen flame ionization detector was used. Argon was carrier gas at 125 ml per min for long chain bases and fatty acid esters, 100 ml per min for inositol, and 60 ml per min for sugars. A column (6 ft × 4 mm) of OV-17 on 60/80 mesh Gas-chrom Q (Applied Science Laboratories) was used at 200° for estimation of inositol and sugars and at 235° for fatty acid methyl esters. The same size column packed with OV-1 on 100/120 mesh Gas-chrom Q (Applied Science Laboratories) was used at 228° for silylated long chain bases and at 178° for silylated long chain alcohols.

Inositol Assay—Identification and measurement of inositol were by a modification of the gas chromatographic method of Wells *et al.* (10). The dried lipid samples and myoinositol standards (Fisher Scientific Co.) were hydrolyzed individually in 0.5 ml of 6 N HCl in polyethylene tubes (Curtin Scientific Co.) placed inside glass tubes which were then sealed. After 40 hours at 105°, lipid was removed with 1 ml of $CHCl_3$. The aqueous layer was removed and the $CHCl_3$ re-extracted with 1.0 ml of water. The combined aqueous extracts were dried in a stream of N_2 and redissolved in 1 ml of H_2O. Aliquots containing 15 to 50 μg of inositol were added to a 1 ml column of AG 1-X1 (OH^-) resin (Bio-Rad Laboratories) and eluted with 10 ml of H_2O. After drying, the eluates were silylated by adding 200 μl of reagent, pyridine-hexamethyldisilazane-trimethylchlorosilane (10:2:1, v/v) (11). The tubes were incubated at 60° for 30 min and vigorously mixed until cool. Identical aliquots of samples were analyzed by gas chromatography, peak height varying linearly with the amount of inositol. Very erratic results were obtained when the hydrolysis was carried out in glass tubes.

Other Analytical Procedures—Carbon, hydrogen, and nitrogen analyses were made by Galbraith Laboratories, Inc., Knoxville, Tennessee. Phosphorus was determined after perchloric acid digestion according to Bartlett (12). P_i was measured without digestion. Sodium was measured by flame photometry after perchloric acid digestion.

Hexose was measured by the phenolsulfuric acid method using mannose as standard (13). For sugar identification the lipid was hydrolyzed at 100° for 2 hours in 1.5 ml of 2 N HCl. The hydrolysate was extracted with 1 ml of $CHCl_3$. One milliliter of H_2O was used to re-extract the $CHCl_3$. The hydrolysate was neutralized by mixing with AG 1 (HCO_3^-) resin (Bio-Rad Laboratories). The neutralized mixture was applied to a small column of the same resin and eluted with water. Portions of the eluate containing approximately 0.5 μmole of sugar were dried and analyzed by gas-liquid chromatography under the same conditions as employed for inositol assay except that silylation was accomplished at room temperature.

Hexuronic acid was measured by the carbazole method with glucuronic acid as standard (14). Hexosamine was assayed according to Rondle and Morgan (15).

Assay of Long Chain Bases and Fatty Acids—The purified lipids were methanolyzed according to Carter and Hirschberg (16) to obtain long chain base and fatty ester fractions. Bases were analyzed by thin layer chromatography on silica gel plates (Quantum Industries) developed with $CHCl_3$-CH_3OH-2 N NH_4OH (40:10:1, v/v) (17) and sprayed with ninhydrin. Standards were sphingenine (sphingosine, Applied Science Laboratories), sphinganine (dihydrosphingosine, Supelco, Inc.), and hydroxysphinganine (phytosphingosine, Calbiochem). Bases were derivatized with the improved silanes reagent of Carter and Gaver (18) and analyzed by gas chromatography.

Long chain bases were quantitatively determined by a modification[2] of the method of Siakotos *et al.* (19). Dried base samples were suspended in 0.05 ml of a methanol solution containing 1.25% (w/v) Brij 35 (Atlas Chemical) and 3.75% (w/v) Brij 30. After adding 0.95 ml of 14% BF_3 in CH_3OH (Sigma Chemical Co.), 3 ml of a buffered hyamine solution (1 M $NaHCO_3$, 0.2 M Na_2CO_3 containing 2.5% 1 M hyamine hydroxide) was added along with 0.5 ml of 0.1% aqueous 2,4,6-trinitrobenzene-sulfonic acid (Eastman Kodak Co.). The tubes were mixed well and incubated in the dark for 1 hour. One milliliter of 1.2 N HCl and 2 ml of benzene were then added and the tubes mixed well and centrifuged. The absorbance of the upper phase was read at 420 nm. Hydroxysphinganine was used as the standard.

Methylation of fatty acids was ensured by treating the petroleum ether extract of the Carter-Hirschberg hydrolysate with 2,2-dimethoxypropane (Dow Chemical Co.) (20). The fatty acid methyl esters were chromatographed on silica gel plates (Quantum Industries) developed with benzene-$CHCl_3$-H_3CCOOH (90:10:1, v/v). Zones were detected with rhodamine. For gas chromatographic identification the esters were silylated with the same reagent used for carbohydrates. Saturated fatty acid standards were OH:22 (Applied Science Laboratories), OH:24 (Applied Science Laboratories), OH:26 (Lachat Chemicals), and C-26 (Supelco, Inc.). A mixture of normal, straight chain, and α-hydroxy fatty acid methyl esters (C-20, C-22, C-24) was obtained from Applied Science Laboratories. Acyl ester was assayed by the ferric hydroxamate method (21).

Periodate Oxidation of Intact Lipids—A slight modification of the method of Carter and Hirschberg (16) was employed to form long chain aldehydes from periodate oxidation of intact hydroxysphinganine containing lipids and reduce these to long chain alcohols prior to gas chromatographic analysis. To the lipid sample (0.1 to 0.5 μmole) dissolved in 0.35 ml of $CHCl_3$-CH_3OH-H_2O (16:16:5, v/v) was added 0.35 ml of 0.057 M sodium metaperiodate in the same solvent. After standing at room temperature in the dark overnight 4 ml of petroleum ether (30°–80°) and 2 ml of water were added and the aldehydes were extracted into the upper phase. This phase was dried, redissolved in 0.5 ml of CH_3OH, and reduced with 0.5 ml of $NaBH_4$ reagent (1 g of $NaBH_4$ in 10 ml of 0.04 N NaOH). After 30 min, 0.03 ml of 1 N HCl, 0.52 ml of H_2O, 0.3 ml of CH_3OH, and 1.6 ml of $CHCl_3$ were added and the mixture shaken well. The $CHCl_3$ phase was dried and the trimethylsilylethers formed by the addition of 0.2 ml of reagent, pyridine-hexamethyldisilazane-trimethylchlorosilane (2:2.6:1.6, v/v). Saturated C-12, C-14, C-16, and C-18 alcohols (Applied Science Laboratories) were used as reference standards for gas chromatography. Some samples were oxidized in the presence of a mixture of alcohols as internal retention time standards. Hydroxysphinganine was run in parallel as a standard and the pentadecanol obtained as expected was used for quantitation.

RESULTS

Preparation of Sphingolipid Concentrate—The preparation of a sphingolipid concentrate is outlined in Fig. 1 and is given in detail under "Experimental Procedures." The method employed for the initial extract has been shown to completely remove radioactivity from cells uniformly labeled with [2-^{3}H]myoinositol (1). This extract is heavily contaminated with non-lipid impurities.

The procedure for the subsequent isolation steps was greatly facilitated by including tracer amounts of the ^{32}P-labeled lipid whose isolation in macroscopic quantities was being attempted. When the ^{32}P-labeled nondeacylatable lipid fraction was subjected to thin layer chromatography the pattern shown in Fig. 3 was found. The ^{32}P-labeled spot with the highest R_F was further purified and added to the crude lipid extract; as will be seen, this substance is an inositol phosphorylceramide.

The next step in the isolation is the nearly quantitative pre-

[2] K. Kaul and R. L. Lester, unpublished experiments.

TABLE I

Phosphorus distribution during lipid purification and recovery of [^{32}P]inositol phosphorylceramide added to crude lipid extract

Purification stage[a]	Total phosphorus	[^{32}P]Inositol phosphorylceramide		
		Total ^{32}P	Recovery	Specific activity
	mmoles	*cpm*	%	*cpm/ μmole P*
1. Lipid extract.....	32.25	157,312	100.0	4.9
3A. Petroleum ether-soluble.....	6.08	134,867	85.5	22.2
4A. Ethanol precipitate.	2.58	120,180	76.1	46.6
5. Chelex eluate.....	2.51	117,175	74.3	46.6
6A. Supernatant.....	1.69	108,260	68.6	64.3

[a] Fig. 1 and "Experimental Procedures."

cipitation of the desired lipid (Table I) by lowering the pH and allowing the mixture to stand in the cold. At this stage the precipitate can be processed by two alternative procedures. The first procedure described in detail involved further purification by means of differential solubility (Stages 3A and 4A) so that by Stage 5, the product was almost 10-fold purified over the starting material with only a 25% loss. Assay of the material at Stage 5 showed that 78% of the lipid P remained nondeacylatable when subjected to mild alkaline methanolysis as described under "Experimental Procedures."

The alternative procedure employed was to dissolve the Stage 2 precipitate in $CHCl_3$-CH_3OH-H_2O (16:16:5, v/v) leaving undissolved non-lipid impurities. The addition of a ½ volume of CH_3OH at room temperature precipitated the most polar sphingolipid. When the resulting supernatant fraction was stored at −20° for several days, the less polar sphingolipids came out of solution in very high yield and provided material suitable for column chromatography after conversion to the Na^+ form.

Fig. 3 is a schematic representation of the results of thin layer chromatography at several stages in the preparation of the sphingolipid concentrate. It can be seen that the preparation at Stage 4A is composed of two glycolipds in the region labeled $M(IP)_2C$. In a future communication it will be shown that these substances both have the composition mannose (inositol P)$_2$ ceramide and that they differ in the nature of the ceramides. These substances are largely removed by methanol precipitation in the next purification stage leaving at Stage 6A several lipids clustered at a higher R_F, in the region of the least polar nondeacylatable ^{32}P lipids. Thus, by relatively mild and simple procedures, a concentrate of the desired lipids (Table I and Fig. 3) was obtained in high yield. Two of these lipids give a positive test when sprayed with the orcinol-H_2SO_4 reagent indicating that they are glycolipids, the upper one being more intense.

Final Purification by Column Chromatography—Preparative thin layer chromatography appeared to be a feasible method to purify these lipids as judged by the results shown in Fig. 3. However, it was found that good thin layer resolution could only be achieved with very light loads, less than 50 nmoles per cm on a 0.5-mm layer. Therefore, an adsorbent was sought with a very high specific surface area to carry out column chromatography. Porasil (porous silica beads) treated with NaOH (which presumably converts some of the silicic acid to sodium silicate) was found to have desirable adsorptive properties. The substances of interest in a small volume could be adsorbed to a column equilibrated with $CHCl_3$ and could be eluted with mixtures of $CHCl_3$, CH_3OH, and H_2O in the same order as their R_F values on thin layer chromatograms (Fig. 3). These columns could be recycled and used many times without repacking.

Stage 6A lipid was first chromatographed on a rather heavily loaded column (2 × 94 cm) at room temperature with the results shown in Fig. 2, *upper panel*. Appropriate fractions were pooled based on elution of internally added ^{32}P reference lipids and on the results of thin layer chromatograms. The least abundant glycolipid (Fig. 3) was found in an impure form with the solvent change at about the 5-liter mark (Fig. 2, *upper panel*) and was not further investigated. The pooled fractions (*IPC*, *IPC + MIPC*, Fig. 2, *upper panel*) were further purified on a column of the same dimensions (not shown). The intermediate fractions thus obtained were impure; therefore, a system of somewhat higher resolving power was employed. The desired purification could be achieved with 200-cm long columns run at 42° (Fig. 2, *middle* and *lower panels*). The intermediate pooled fractions, designated IPC, from the first two shorter preparative columns gave rise to two distinct phosphorus-containing peaks, IPC-I and IPC-II, when subjected to chromatography on the longer columns at 42° (Fig. 2, *middle panel*). After evaporation of solvent the material from these peaks was redissolved and precipitated as indicated under "Experimental Procedures" and the resulting products will be referred to as IPC-I and IPC-II.

The other intermediate pooled fraction (*IPC + MIPC*, Fig. 2, *upper panel*) upon rechromatography at 42° on a 200-cm column gave the results shown in the *lower panel* of Fig. 2. The substances hereafter referred to as IPC-III and MIPC were imperfectly resolved; however, the fractions pooled as indicated were not cross-contaminated as judged by thin layer chromatography.

Purity of Isolated Lipids—The four lipids thus isolated, IPC-I, IPC-II, IPC-III, and MIPC were subjected to thin layer chromatography and compared to the total ^{32}P-labeled nondeacylatable lipid fraction (Fig. 3, *right panel*). Each individual lipid gave a single rhodamine-positive, ninhydrin-negative spot. Only MIPC gave a positive reaction with the orcinol-H_2SO_4 spray and was at the exact R_F of the upper glycolipid spot observed in the sphingolipid concentrate (Fig. 3). IPC-III could be seen to have a very slightly higher R_F than MIPC; however, a mixture of these two for all practical purposes evidenced a single spot. IPC-I had an R_F definitely higher than that of IPC-II. In the original mixture IPC-II is present in much higher quantities than IPC-I and in these ratios a complete separation is rarely observed with this thin layer chromatography system which is very sensitive to overloading.

The purity of IPC-I and IPC-II and the validity of the column chromatography procedure was further checked by chromatography of small amounts of each of these substances on 300-cm Porasil columns. Included in each sample was the same labeled marker, purified from the major high R_F spot on thin layer chromatograms of a nondeacylatable lipid fraction. The results in Fig. 4 show that both IPC-I and IPC-II chromatograph as a single phosphorus-containing peak. IPC-II, the major lipid purified, coincides perfectly with the labeled marker; however, IPC-I clearly emerges from the column ahead of the marker.

Thus, by chromatographic criteria, the four compounds appear to be homogeneous and distinct. The methodology at hand would not be expected to detect 10% cross-contamination between IPC-I and IPC-II. IPC-III and MIPC are both well enough separated from IPC-II by thin layer chromatography to rule out the presence of IPC-II in samples of IPC-III or MIPC. Although MIPC and IPC-III do not separate well by thin layer chromatography, MIPC contamination of IPC-III could readily be detected because only MIPC reacts with glycolipid sprays.

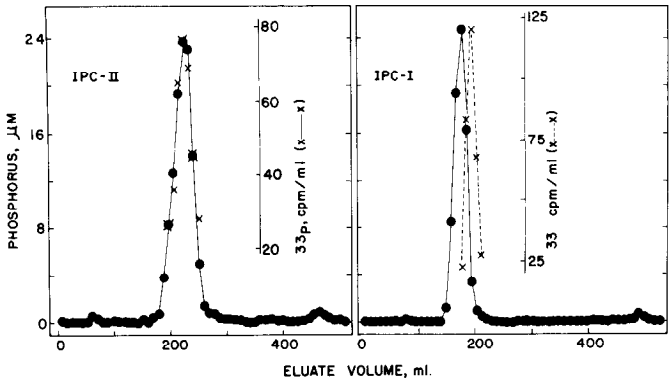


FIG. 4. Analytical column chromatography of purified inositol phosphorylceramides I and II (*IPC-I, -II*). To each column was added some [^{33}P]inositol phosphorylceramide II (5000 cpm) as internal reference marker prepared essentially as described under "Experimental Procedure" for the ^{32}P-labeled lipids. The specific activity was not measured but the amounts added did not contribute significantly to the chemical P analyses. Inositol phosphorylceramide-II (1.02 μmoles of phosphorus, *left panel*) and inositol phosphorylceramide I (0.66 μmole of P, *right panel*) were dissolved in 0.6 ml of Solvent A and added to columns (0.6 × 300 cm) of Porasil. Elution was carried out at 42° with 360 ml of $CHCl_3$-CH_3OH-H_2O (65:37:2.7, v/v) at a flow rate of 1.2 ml per min followed by 188 ml of Solvent A at a flow rate of 2.51 ml per min. From each 4.5-ml fraction 1 ml was counted (×——×) in a Triton-containing scintillation fluid (1) and the rest of the sample was used for total phosphorus assay (●——●). Recovery of phosphorus for each column was 105%.

TABLE II

Elemental composition of purified inositol phosphorylceramide fractions expressed as per cent of dry weight

Fraction	C	H	N	P	Na
	%				
IPC-I + IPC-II	60.07	10.37	1.39	3.17	2.47
IPC-II	59.72	9.57	1.35	2.93	2.44
IPC-II[a]	61.51	10.22	1.44	3.18	2.36
IPC-II[a]·$2H_2O$	59.32	10.26	1.38	3.06	2.27

[a] Calculated values assuming the empirical formula, $C_{50}H_{99}O_{13}NPNa$, for a compound with equimolar amounts of inositol, phosphorus, hydroxyhexacosanoic acid, and hydroxysphinganine.

IPC-III contamination of MIPC preparations would, however, be impossible to detect by the thin layer chromatographic systems thus far tested.

Chemical Composition of Four Purified Lipids—The final preparation of IPC-II had equimolar amounts of phosphorus, nitrogen, and sodium (1.0:1.04:0.94, Table II). This showed no significant difference from the mixture of IPC-I plus IPC-II applied to the final chromatography column (phosphorus-nitrogen-sodium, 1.0:0.96:1.05). The equivalent weights based on nitrogen, phosphorus and sodium analysis of IPC-II were 1038, 1057, and 942, respectively; the expected equivalent was 975.3 based on the assumptions stated in Table. II.

Each of the four substances isolated was clearly an inositol-containing phosphosphingolipid since each contains equimolar amounts of phosphorus, inositol, fatty acid, and long chain base (Table III). In addition, compound MIPC showed 1 hexose eq per phosphorus (Table III). Mannose was identified by gas-liquid chromatography as the sole carbohydrate in an acid hydrolysate of MIPC. Tests on all four compounds for uronic acid and hexosamine were negative.

TABLE III

Quantitative analyses of purified sphingolipids

Data are reported as moles per mole of total phosphorus.

Lipid	Inositol	Hexose	Fatty acid[a]	Long chain base
IPC-I	1.01	<0.06	1.1	0.96
IPC-II	1.00	<0.06	1.1	0.90
IPC-III	0.91	<0.02	0.7	0.93
MIPC	1.09	1.04	1.1	1.1

[a] Assayed as total ester after HCl-methanolysis.

TABLE IV

Identification of long chain base fractions obtained from methanolysis of sphingolipids

Details of the gas-liquid chromatography procedure are given under "Experimental Procedures."

Lipid	Peak	Tentative designation	Total detector response	Retention time	
				Found	Expected[a]
			%	*min*	
IPC-I	1	Hydroxysphingenine	87	7.5	7.5
	2	Hydroxyeicosasphingenine	13	14.0	14.0
IPC-II	1		5	4.1	
	2	Hydroxysphinganine	93	8.1	8.1
	3	Hydroxyeicosasphinganine	2	15.1	15.1
IPC-III	1		11	4.1	
	2	Sphinganine	1	5.3	5.3
	3	Hydroxysphinganine	88	8.1	8.1
MIPC	1	Sphinganine	91	5.3	5.3
	2		4	8.8	
	3	Eicosasphinganine	5	9.8	9.8

[a] Values obtained from sphinganine and hydroxysphinganine standards or extrapolated from experimental data given by Carter and Gaver (18).

The long chain base fractions from acid hydrolysis were analyzed by thin layer chromatography. The base fractions from IPC-I, IPC-II, and IPC-III all gave single ninhydrin positive spots at the R_F of the hydroxysphinganine (phytosphingosine) reference standard. The long chain base fraction of MIPC gave a single spot with an R_F identical with sphinganine (dihydrosphingosine). Gas chromatography of the trimethylsilylether derivatives of the long chain base fractions gave results (Table IV) consistent with those from thin layer chromatography. The principal base in MIPC was sphinganine and in IPC-II and IPC-III it was hydroxysphinganine. The principal peak found in the IPC-I base fraction had the retention time of hydroxysphingenine (dehydrophytosphingosine) with lesser amounts of hydroxyeicosasphingenine. The expected retention times for the IPC-I bases were extrapolated from the retention times of the available standards and the data of Carter and Gaver (18).

The fatty acid methyl ester fractions from each lipid were subjected to thin layer chromatography and each gave one predominant spot detected with the rhodamine spray. The major spot in IPC-I and MIPC migrated with the R_F of a mixture of methyl esters of C-14 to C-26 acids and the only spot in the IPC-II fraction had the R_F of the methyl esters of hydroxy acids with 16 to 26 carbons. The single spot from the fatty acid ester of

TABLE V

Identification of the fatty acid esters obtained by methanolysis of sphingolipids

The methyl esters were silylated and resolved by gas-liquid chromatography as given under "Experimental Procedures."

Lipid	Peak	Identity	Total detector response	Retention Time	
				Found	Expected[a]
			%	*min*	
IPC-I	1	?	5	6.0	
	2	26	74	19.0	19.0
	3	OH:26	21	32.2	32.2
IPC-II	1	?	3	6.0	
	2	OH:24	1	16.6	16.6
	3	OH:26	96	32.2	32.2
IPC-III	1	?	5	6.0	
	2	26	2	19.0	19.0
	3	OH:26	2	32.2	32.2
	4	diOH:26	91	45.0	45.0
MIPC	1	?	5	6.0	
	2	26	75	19.0	19.0
	3	OH:26	20	32.2	32.2
	4	diOH:26	1	45.0	45.0

[a] Values obtained from standards.

IPC-III had an R_F of 0.05 as compared to an R_F of 0.22 for the monohydroxy fatty acid esters. The methyl esters were silylated and subjected to gas chromatography. The results (Table V) are wholly consistent with the thin layer data and show all the principal components to have retention times corresponding to authentic standards. Almost all the fatty acids are 26 carbons long.

The principal component of IPC-III has the same retention time as the trimethylsilyl derivative of methyl-2,3-dihydroxyhexacosanoic acid, samples of which were generously provided by Dr. M. Proštenik and by Dr. Y. Kishimoto. These workers and their colleagues (22, 23) recently demonstrated the occurrence of this substance in ceramide and ceramide phosphate preparations from autolyzed yeast.

Structural Studies with IPC-II—Hydrolysis of IPC-II by aqueous acid or alkali converted almost all the phosphorus to a water-soluble organic form. This product appeared to be an inositol monophosphate since after treatment with the *Escherichia coli* alkaline phosphatase equimolar amounts of P_i and free inositol were formed (Table VI). This experiment strongly indicates that phosphoinositol is a component of IPC-II.

IPC-II was treated with periodate as outlined under "Experimental Procedures." The products were extracted, reduced, silylated, and subjected to gas chromatography. IPC-II gave products with the precise retention times of trimethylsilylpentadecan-1-ol and trimethylsilylheptadecan-1-ol and in a yield equivalent to 100% of the detector response obtained with equimolar amounts of hydroxysphinganine run as a parallel standard. This result indicates that the 3 and 4 hydroxyl groups of the long chain base in IPC-II are unsubstituted.

To get evidence concerning the linkage of the phosphoinositol group to the ceramide in IPC-II, an attempt was made to identify the polar products resulting from the oxidation of IPC-II with periodate followed by reduction with [^{3}H]$NaBH_4$. If the phosphoinositol were linked to the 1-hydroxyl of the long chain base then the principal tritium containing lipid products of such a degradation would be a long chain alcohol and more polar products such as *N*-fatty acyl-glycerophosphorylserinol and possibly some *N*-fatty acyl-phosphoserinol. Since the presumed phosphodiester bond could be cleaved on either side, these polar lipids would be expected to yield as water-soluble products after HCl hydrolysis ^{3}H-labeled glycerophosphate, serinol, glycerol, and phosphoserinol. The presence of phosphoserinol would be strong evidence that inositiol phosphate is linked via a phosphodiester to the C-1 hydroxyl of the hydroxysphinganine. IPC-II was oxidized with periodate and then treated with [^{3}H]$NaBH_4$ and the resulting reaction mixture was separated by thin layer chromatography as described in the legend to Fig. 5. The polar lipid spots were eluted, pooled, and hydrolyzed with butanol-HCl. About 75% of the polar lipid radioactivity was rendered water-soluble by this treatment. This water-soluble fraction was subjected to anion exchange column chromatography and four radioactive fractions were pooled as indicated in Fig. 5.

The identity of the radioactivity in each fraction (Fig. 5) was examined by paper chromatography with two different developing solvents before and after treatment with alkaline phosphatase (Table VII). Fraction I, which represents material not bound to the anion exchange resin, contained radioactivity with the precise R_F of serinol and glycerol in both chromatography systems. The amount of glycerol recovered is probably underestimated (Table VII) since processing of the samples required solvent evaporation at several steps. Fraction II was not identified; radioactivity was distributed equally in two broad zones

TABLE VI

Analysis of water-soluble hydrolysis products of IPC-II

Basic hydrolysis was carried out by treating a suspension of ICP-II (1.02 μmoles of P) in 0.25 ml of 1.0 N aqueous KOH at 37° for 15 hours. The mixture was neutralized with Bio-Rex 70 (H^+) resin (Bio-Rad Laboratories), filtered through glass wool and the resin rinsed with water. Aliquots of this eluate were sampled for total and inorganic phosphorus as described under "Experimental Procedures." The amount of free inositol in this eluate was determined by gas chromatography as described under "Experimental Procedures" except that silylation was carried out directly on a dried sample with 0.3 ml of reagent and heated at 100° overnight. *E. coli* alkaline phosphatase (250 μg, Worthington) was added to a portion of the eluate in 1 ml of 0.02 N ammonium acetate, pH 8.6. After incubating at room temperature overnight the mixture was assayed for total phosphorus, P_i, and free inositol. Acid hydrolysis was accomplished by incubating IPC-II (1.02 μmoles of P) in 0.5 ml of 2 N HCl for 3 hours at 100°. After the addition of 0.5 ml of H_2O and 0.5 ml of $CHCl_3$, the mixture was shaken, centrifuged, and the upper layer removed. The $CHCl_3$ was re-extracted with 1 ml of H_2O, and the pooled aqueous fractions were dried under nitrogen and redissolved in H_2O. A portion was subjected to alkaline phosphatase treatment as above for 3 hours. Assays for total phosphorus, P_i, and free inositol were carried out on samples before and after phosphatase treatment. Inositol was assayed as under "Experimental Procedures" except that the 6 N HCl hydrolysis step and $CHCl_3$ extractions were omitted.

Hydrolysis procedure	Initial lipid P	Water-soluble products				
		Before phosphatase			After phosphatase	
		Total P	P_i	Free inositol	P_i	Free inositol
	μmoles					
1 N KOH, 15 hours, 37°	1.02	1.05	≤0.04	0.06	1.01	0.99
2 N HCl, 3 hours, 100°	1.02	0.88	0.06	0.01	0.98	0.80

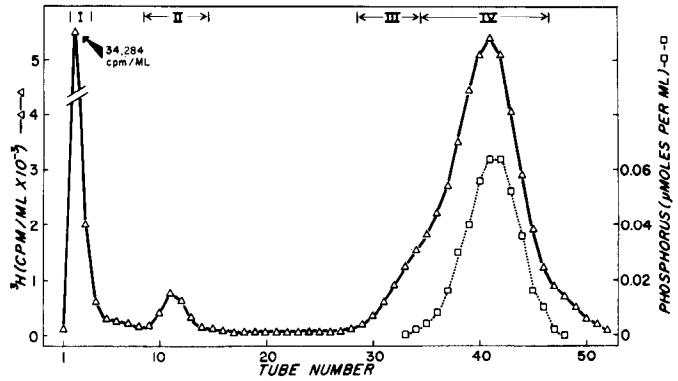


FIG. 5. Anion exchange chromatography of some degradation products of IPC-II. IPC-II (0.49 μmole of phosphorus) was reacted with 0.35 ml of 0.029 M $NaIO_4$ in Solvent A for 4 days in the dark. [^{3}H]$NaBH_4$ (200 μmoles, approximately 41 mCi per mmole, New England Nuclear Corp.) dissolved in 1 ml of 0.05 N NaOH in water was added to the reaction mixture followed by 1.5 ml of CH_3OH. After 1 hour at 63°, 1 ml of acetone was added to react with the excess borohydride for 15 min at room temperature. Isopropyl alcohol (5 ml) was added and the mixture was distilled *in vacuo;* this was repeated twice more to remove the excess [^{3}H]-isopropyl alcohol. The dried product was dissolved in a mixture of 0.35 ml of Solvent A, 1 ml of H_2O and 1.5 ml of CH_3OH. A thick slurry of Dowex 50 (H^+) (1.5 ml equilibrated with the same solvent mixture as the sample) was added to the sample and the mixture was poured through a 1-ml column of additional Dowex 50 (H^+) and rinsed twice with 2 ml of Solvent A. The eluate and rinses were taken to dryness *in vacuo*. Methanol was added and the solvent was evaporated; this was repeated twice to remove excess boric acid as methyl borate. The sample was dissolved in Solvent A and applied to a silica gel thin layer plate and developed as in Fig. 3. Radioactivity was detected at the solvent front where the expected long chain alcohol migrates as well as in the region with an R_F of 0.60 to 0.84. This polar lipid fraction was eluted with Solvent A (2.8×10^6 cpm) taken to dryness and treated for 30 min at 100° with 1 ml of 3 N HCl in 1-butanol-H_2O (1:1). The products were partitioned after the addition of 2 ml each of $CHCl_3$ and H_2O. The aqueous phase containing 2.0×10^6 cpm was taken to dryness several times to remove HCl and finally dissolved in water adjusted to pH 8 with NH_3. This sample along with 4 μmoles of *sn*-glycero-3-phosphate were applied to a column (0.6 × 81 cm) of AG 1X-2, 200 to 400 mesh resin (Bio-Rad Laboratories) in the HCO_3^- form which had been equilibrated with 60 ml of 0.1 M NH_4HCO_3 pH 7.85 and then with 30 ml of H_2O. Elution was carried out with 20 ml of H_2O followed by 0.1 M NH_4HCO_3 pH 7.85 at a flow rate of 1.27 ml per min. Fractions (9.14 ml) were collected and an aliquot of each was assayed for ^{3}H and total phosphorus.

(R_F 0.40 to 0.65, R_F 0.65 to 0.90) with Solvent II (Table VII) and no change in R_F was observed after phosphatase treatment. Fraction III gave a major spot at R_F 0.15 and a minor spot at R_F 0.26 (Solvent II), the latter being the precise R_F of glycero-3-phosphate. After phosphatase treatment, both of these spots disappeared and radioactivity was found at the precise R_F values (with both solvents) of glycerol and serinol as indicated in Table VII. Although an authentic standard for phosphoserinol was not available, the evidence is strong that the major component of Fraction III constituting >95% of the ^{3}H is *o*-phosphoserinol. It can be seen that the bulk of the radioactivity of Fraction IV corresponds to the retention time of glycero-3-phosphate on the anion exchange column (Fig. 5). Paper chromatography disclosed that about a sixth of the ^{3}H in Fraction IV had the R_F of the major component of Fraction III, that is, phosphoserinol. In both paper chromatography systems the major component had the exact R_F of *sn*-glycero-3-phosphate. These systems just barely resolve glycero-2-phosphate from glycero-3-phosphate (1). Because of the closeness of R_F values, it cannot be excluded

TABLE VII

Distribution and identification of water-soluble labeled products resulting from degradation of IPC-II

Eluates were pooled as indicated in Fig. 5 (Fraction I, tubes 1 and 2; Fraction II, tubes 9 to 14; Fraction III, tubes 29 to 34; Fraction IV, tubes 35 to 46). Fraction I was taken to dryness directly. Fractions II to IV were treated with Dowex-50 (H^+) to remove NH_4^+ and then taken to dryness *in vacuo*. Each fraction was chromatographed on 589 Orange-Ribbon paper (Scheicher and Schuell) with two different solvents with appropriate standards including [^{14}C]glycerol which was subsequently detected by scintillation counting. Solvent I, isobutyric acid-H_2O-concentrated NH_4OH (66:33:1 v/v), gave the following R_F values: glycero-3-phosphate, 0.29; phosphoserinol, 0.35; glycerol, 0.53; serinol, 0.67. Solvent II containing EDTA, NH_4HCO_3, H_2O and CH_3CH_2OH (37) gave the following R_F values: phosphoserinol, 0.15; glycero-3-phosphate, 0.26; serinol, 0.60; glycerol, 0.72. The R_F of the major component of Fraction III is taken as that of phosphoserinol since an authentic standard was not available. The radioactivity in each lane was located by cutting up the paper and counting each piece in a Triton-toluene-H_2O-containing counting fluid (1). Serinol was located with ninhydrin and glycerophosphate was detected with periodate. In each case the ^{3}H was at the precise R_F of the authentic compounds. Fractions III and IV were treated with alkaline phosphatase (3) and the reaction mixture was spotted directly and developed with Solvent II. Paper chromatography with Solvent I is rather sensitive to buffer so the phosphatase reaction mixture of Fraction III was first passed through a small column of Dowex 1 (HCO_3^-) and then concentrated and chromatographed. All the ^{3}H in the reaction mixture was recovered after this treatment. After phosphatase treatment all the ^{3}H products gave the precise R_F of serinol or glycerol in both chromatography solvents with the recoveries as indicated. Each chromatographic lane contained 3 to 10 × 10^3 cpm of ^{3}H. The data are reported as the total counts per min recovered in each fraction for the experiment described in the legend to Fig. 5.

Fraction	Location of tritium after paper chromatography			
	Glycerol	Serinol	Glycero-phosphate	Phos-phoserinol
	cpm/total fraction			
I	175,000	553,000	0	0
III	0	0	3,500	85,100
III + phosphatase	3,100	78,900	0	0
IV	0	0	620,800	118,200
IV + phosphatase	606,000	133,000	4,400	0

that as much as 10 to 15% of the total glycerophosphate radioactivity is present as glycero-2-phosphate. After treatment of Fraction IV with phosphatase, all the radioactivity was recovered as serinol and glycerol in the amounts expected if the original substances were glycerophosphate plus phosphoserinol. Of the lipid bound serinol about one-quarter was recovered in a phosphorylated form (Table VII). It would appear that the predominant product of the periodate-borohydride degradation was *N*-fatty acyl-glycerophosphorylserinol. The yield of the various labeled products (Table VII) is consistent with the notion that this compound should give upon hydrolysis more glycerophosphate than phosphoserinol due to the possibility of the formation of cyclic glycerophosphate as an hydrolysis intermediate.

The infrared spectrum (KBr) of IPC-II showed strong bands at 1640 cm^{-1} and 1525 cm^{-1} expected for a secondary amide and no bands typical of carboxylic esters.

Quantitative Distribution of All Phospholipids Containing

Inositol—Having established the essential and distinguishing chemical characteristics of all the inositol-containing phospholipids, it is of interest to measure the relative amounts of each in the yeast cell. The total lipid extract from cells uniformly labeled with [2-^{3}H]myoinositol and $^{32}P_i$ was prepared. Earlier work has demonstrated that all the trichloroacetic acid-insoluble ^{3}H from such cells can be extracted by the procedure employed; furthermore, it was shown that all of the ^{3}H in this lipid extract could be recovered as [^{3}H]inositol after strong acid hydrolysis (1). This extract was subjected to two-dimensional chromatography on silica gel-impregnated paper (4) and all ^{32}P-labeled zones were counted with the results shown in Table VIII. It can be seen that the inositol containing sphingolipids constitute 40.9% of the total lipid inositol. A third of the sphingolipid inositol is found in those sphingolipids with a single inositol phosphate group.

The crude lipid extract was subjected to a deacylation procedure and the resulting nondeacylatable fraction was chromatographed in the same manner. The results expressed as radioactivity per unit cell mass (Table VIII) shows essentially quantitive recovery of ^{32}P and ^{3}H in the two sphingolipid zones with a complete loss of radioactivity in the other lipid zones.

The monophosphoinositol-containing sphingolipids are reported as a mixture in Table VIII because the compounds are imperfectly resolved with this chromatographic system. IPC-III and the MIPC isolated are not separated in this system. As is developed under "Discussion," it was of interest, however, to assess how much glycolipid is present in this mixture of monophosphoinositol-containing, nondeacylatable lipids. Therefore, a nondeacylatable lipid fraction was prepared from a total nonradioactive lipid extract by this procedure which results in quantitive recovery of all the inositol-containing phosphosphingolipids (Table VIII). This nondeacylatable lipid fraction was chromatographed on a Porasil column with an elution schedule that separated the monophosphoinositol sphingolipids from the diphosphoinositol containing sphingolipids as judged by thin layer chromatography of the eluates (Table IX). A quantitative hexose assay was performed on the key fractions. The hexose to phosphorus ratio indicates that the amount of glycolipid in the monophosphoinositol sphingolipid fraction is only 15%; thus, the inositol phosphorylceramides are the major components.

An important consideration underlying the interpretation of the data given in Tables VIII and IX is whether mannosylinositol phosphorylceramide could have been artifactually formed from mannosyldiinositol diphosphorylceramide during the deacylation procedure. Under some conditions alkali has been shown to cause this conversion with the release of free inositol phosphate (6). Therefore, we exposed some pure mannosyldiinositol diphosphorylceramide to the same deacylation procedure and found no more than 0.9% of the phosphorus became water-soluble. This control experiment shows that no more than 10% of the glycolipid found in the monophosphoinositol sphingolipids fraction (Table IX) could have come from breakdown of the more polar sphingolipid due to the deacylation procedure.

DISCUSSION

The data presented herein and that published earlier (1, 5, 6) establish the chemical nature of all quantitatively significant inositol-containing lipids in *S. cerevisiae*. The current work has shown that the heretofore unidentified nondeacylatable inositol-containing phospholipids (5) are a group of closely related sphingolipids. The spot previously designated 7 (5) has been shown to be a mixture of substances, IPC-I and IPC-II. Spot

TABLE VIII

Relative distribution of inositol and phosphorus containing lipids in total lipid extract before and after deacylation

Cells were uniformly labeled with $H_3{}^{32}PO_4$ and [2-^{3}H]myoinositol and lipid fractions were prepared as described under "Experimental Procedures." Equivalent amounts of each fraction were subjected to two-dimensional chromatography and ^{32}P zones were detected by autoradiography and counted as described under "Experimental Procedures." The lipid radioactivity spotted in the total extract contained 71,500 cpm of ^{32}P and 70,000 cpm of ^{3}H.

Lipid zone[a]	Total lipid extract				Nondeacylatable lipid fraction	
	^{3}H	^{3}H[b]	^{32}P	^{32}P	^{3}H	^{32}P
	cpm/mg dry weight	%	*cpm/mg dry weight*	%	*cpm/mg dry weight*	
$M(IP)_2C$	249,500	27.9	58,500	8.9	209,600	63,200
DPI	5,400	0.6	2,780	0.4	0	0
IPC + MIPC	116,300	13.0	31,400	4.7	121,000	32,150
MPI	522,000	58.2	151,000	22.8		
All other phospholipids	2,700	0.3	416,000	63.1	895[c]	2,385[c]

[a] Abbreviations are: $M(IP)_2C$, mannosyldiinositol diphosphorylceramide; DPI, diphosphoinositide; MPI, phosphatidylinositol.

[b] Percent total ^{3}H found in spots that have ^{32}P.

[c] This represents the sum of all zones with ^{32}P with R_F values above IPC-I, including phosphatidylinositol.

TABLE IX

Distribution of hexose containing phospholipids stable to mild alkaline methanolysis

A lipid extract, prepared from cells in late log phase, was subjected to mild alkaline methanolysis as described under "Experimental Procedures." An aliquot of the alkali-stable lipid fraction (0.9 ml, 1.98 μmoles of phosphorus) dissolved in Solvent III was added to a column (0.5 × 55 cm) of Porasil packed in chloroform. After washing in the sample with 0.5 ml of chloroform, the column was eluted with the solvents indicated below, collecting 12-ml fractions. Phosphorus and hexose were determined as indicated under "Experimental Procedures." To determine phospholipid composition, silica gel thin layer chromatography was carried out on 0.5 ml of each fraction with the solvent chloroform-methanol-4.2 N NH_4OH (9:7:2, v/v). All lipids were detected by spraying with rhodamine; glycolipids were observed by subsequent treatment with orcinol-H_2SO_4 as described under "Experimental Procedures."

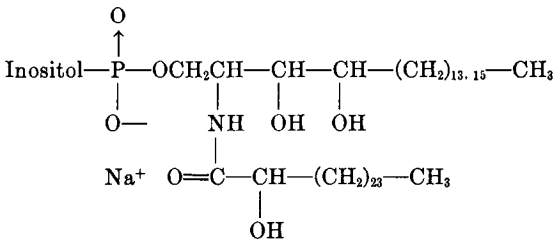
Fraction	Solvent[a]	Volume	Recovery of P	Hexose/P	Phospholipid composition[b]
		ml	%		
1–2	Chloroform	24	0.0		
3–4	I	24	0.0		
5	II	12	0.0		
6	II	12	37.7	0.15	IPC + MIPC
7	II	12	7.5		$M(IP)_2C$
8	III	12	7.5		$M(IP)_2C$
9	III	12	45.3	0.48	$M(IP)_2C$
10	III	12	1.9		

[a] Solvents: I, chloroform-methanol (5:1), v/v); II, chloroform-methanol-water (65:55:6, v/v); III, chloroform-methanol-water (16:16:5, v/v).

[b] Abbreviations: $M(IP)_2C$, mannosyldiinositol diphosphorylceramide.

6 (5) has been shown to be a mixture of substances, IPC-III and MIPC. All four substances isolated appear to be sphingolipids containing equimolar amounts of inositol, phosphorus, fatty acid, and long chain base. Since all these compounds survive mild alkaline methanolysis, it is presumed that the fatty acid is not present in an esterified form but rather as an amide involving the long chain base. Direct evidence on this point is available only with IPC-II which shows infrared absorption bands typical of amides.

Since the substance IPC-II yields all its phosphorus to form an inositol monophosphate after either acid or base hydrolysis (Table VI) and in view of the presence of equimolar amounts of phosphorus, inositol, sodium, fatty acid, and long chain base (Tables II and III) this compound can be formulated as an inositol phosphorylceramide. Periodate oxidation of IPC-II followed by borohydride reduction and hydrolysis gave a significant yield of phosphoserinol and since sodium periodate also cleaves the intact lipid to give a high yield of 15 and 17 carbon fragments, presumably from the long chain base, it is reasonable to conclude that the site of attachment of the phosphoinositol moiety is to the 1-hydroxyl group of the ceramide and that IPC-II can be formulated as the novel substance,



The detailed stereochemistry of the inositol phosphate linkage and hydroxyl groups remains to be demonstrated.

The presence of equimolar amounts of phosphorus, inositol, long chain base, and fatty acid in compounds IPC-I and IPC-II strongly suggests their close relationship to inositol phosphorylceramide II. These three compounds have very similar chromatographic behavior (Figs. 2 to 4) and the differences that do exist are nicely accounted for by differences in the nature of the fatty acids and the long chain bases. Thus, the total number of free hydroxyl groups in the ceramide portion of these lipids is 2, 3, and 4 for IPC-I, -II, -III, respectively, which are in order of decreasing R_F on thin layer chromatograms. It is therefore proposed that IPC-I and IPC-III are also inositol phosphorylceramides.

Hydrolysis of the compound MIPC yields equimolar amounts of mannose, inositol, phosphorus, hexacosanoic acid, and sphinganine. Insufficient material was available for further chemical analysis; however, our working hypothesis is that this substance is a mannosylinositol phosphorylceramide. This substance migrates at the R_F of one of the two glycolipids (Fig. 3) observed in a crude sphingolipid concentrate and which also has the R_F of the earlier designated (5) Spot 6. The additional glycolipid spot observed in the crude concentrate at a slightly lower R_F (Fig. 3) is also nondeacylatable and corresponds to the earlier designated Spot 5 (5). This lipid was present in small amounts (about 1% of the lipid inositol) and we chose not to attempt isolation at this time. However, earlier data on Spot 5 (5) showing the presence of equimolar amounts of labeled inositol and phosphorus and showing its stability to mild alkaline methanolysis coupled to the present data showing it to be a glycolipid lead us to provisionally conclude that Spot 5 may also be a glycosylinositol phosphorylceramide that differs from the mannosylinositol phosphorylceramide isolated in that it has a more polar ceramide moiety.

We believe that the chromatographic methods employed in the purification of these sphingolipids will prove to be useful in establishing the presence of these compounds and related compounds in other natural sources.

The structures proposed above for the yeast lipids relates them to the structure of phytoglycolipid, a group of complex sphingolipids in plants characterized by Carter and co-workers (24) to have the structural features shown here schematically as

glucosamine-glucuronic acid-inositol-phosphate-ceramide
|
mannose

It is quite unlikely that significant amounts of such compounds occur in *S. cerevisiae* since we have now accounted for all inositol-containing lipids that comprise >1% of the lipid inositol.

The close structural similarity among all the yeast inositol containing phosphosphingolipids suggests the possibility that they may be related biosynthetically. In this regard it is noteworthy that the phosphoinositol group of phosphatidylinositol appears to be transferred intact to mannosyldiinositol diphosphorylceramide as judged by *in vivo* turnover experiments (1).

In 1966 Wagner and Zofcsik (25) isolated a substance from *S. cerevisiae* which they called "mycoglycolipid" and characterized as a mannosylinositol phosphorylceramide containing hydroxysphinganine, hydroxyeicosasphinganine, sphinganine, and very long chain hydroxy and nonhydroxy fatty acids. These workers treated the crude lipid extract with 1 N aqueous KOH for 24 hours prior to isolation of this substance. This treatment introduced by Schmidt *et al.* (26) was designed to decompose acyl ester lipids as a step in the analysis of sphingomyelin, noted by Thudichum (27) to be rather base-stable. Subsequent work (6) showed that the major, most polar yeast phosphosphingolipid is completely decomposed by such treatment, releasing a free phosphoinositol moiety, and giving rise to another lipid with a higher R_F with the properties of mycoglycolipid. Thus, it is clear that mannosylinositol phosphorylceramide can be artifactually produced by this treatment. It is also noteworthy that such base treatment also completely destroyed inositol phosphorylceramide II (Table VI) producing an inositol monophosphate. This lability to base contrasted to the stability of sphingomyelin is probably due to the formation of cyclic inositol phosphate(s) as intermediate(s) in the hydrolysis. Such treatment of a crude yeast lipid extract with alkali would therefore completely destroy the major inositol-containing sphingolipids producing mannosylinositol phosphorylceramide as the sole phospholipid product.

In view of the above it is fair to ask whether the small amount of glycolipid we have observed in the nondeacylatable lipid fraction and have characterized tentatively as mannosylinositol phosphorylceramides are in fact naturally occurring substances. One can never categorically exclude the possibility of artifactual formation; however, we feel that the balance of evidence suggests that these compounds occur as such. These compounds are evident in chromatograms of crude lipid extracts that are prepared in a very mild fashion. Furthermore, as shown previously much more rapid incorporation of [^{14}C]2-deoxy-D-glucose (5), [^{14}C]D-mannose,[3] and [^{14}C]D-glucose[3] was observed into 2 lipids with the R_F values of the presumptive mannosylinositol phosphorylceramides than into mannosyldiinositol diphosphoryl-

[3] S. Steiner and R. L. Lester, unpublished experiments.

ceramide. These results are more consistent with the possible biosynthetic formation of the more complex sphingolipid from mannosylinositol phosphorylceramide rather than formation of mannosylinositol phosphorylceramide by artifactual breakdown of the more complex sphingolipid. Thus, we feel our work establishes the natural occurrence of the mannosylinositol phosphorylceramides.

Sphingolipid isolation procedures that involve destruction of acyl ester lipids are valuable if they cause no breakdown of the desired lipids. Mild alkaline methanolysis carried out as described under "Experimental Procedures" achieves this goal.

Ceramide-like substances called cerebrins have been isolated from autolyzed yeast and fungi (28–32). These substances have been shown to commonly contain hydroxysphinganine and a C-26 fatty acid having zero, one, or two hydroxyl groups (22, 23, 30–35). It is clear that the cerebrins could have arisen as autolytic degradation products of the more complex inositol-containing phosphosphingolipids described in this paper and elsewhere.

The presence of very long chain (C_{20}-C_{26}) fatty acids in animal and plant sphingolipids is fairly common. In addition to the work of Wagner and Zofcsik (25) already discussed, more recently Nurminen and Suomaleinen (36) have also claimed the occurrence of very long chain hydroxy and nonhydroxy fatty acids in naturally occuring sphingolipid preparations from yeast. These latter workers obtained a "complex sphingolipid" from preparations of cell envelopes isolated from a commercial strain of *S. cerevisiae*. They obtained this lipid by the procedure of Steiner *et al.* (6) except that the last step was omitted. As originally described (6) this procedure produces pure mannosyldiinositol diphosphorylceramide without resorting to chromatography. In our hands the lipid preparation at the penultimate step was a very rich sphingolipid concentrate not unlike Stage 5 (Figs. 1 and 3), *i.e.* a mixture of the monophosphoinositol- and the diphosphoinositol-containing sphingolipids. In the last step of the published procedure (6) the lipid mixture was partitioned repeatedly with a biphasic mixture of H_2O, isopropanol, and diethyl ether, obtaining the most polar sphingolipid in pure form in the lower phase and the least polar sphingolipids in the upper phase. Nurminen and Suomalainen (36) report a hexose to phosphorus ratio of 1.14 for their complex sphingolipid. In the absence of thin layer chromatographic data it is difficult to know precisely which sphingolipids were present in their preparation. Nevertheless to establish the precise cellular location of each species of the inositol-containing phosphosphingolipids will no doubt be of value in understanding the biological role of these hitherto poorly defined compounds.

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