tributes to cell adhesion and fusion events should be examined in the future.

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Registry No. Cholesterol, 57-88-5.

References

Ahkong, Q. F., Tampion, W., & Lucy, J. A. (1975) *Nature (London) 256*, 208–209.
Bachi, T., Aquet, M., & Howe, C. (1972) *J. Virol. 11*, 1004–1011.
Baker, R. F., & Kalra, V. K. (1979) *Biochem. Biophys. Res. Commun. 86*, 920–928.
Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 446–468.
Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.
Copeland, B. R., & McConnell, H. M. (1980) *Biochim. Biophys. Acta 599*, 95–109.
Fraley, R., Wilschut, J., Düzgüneş, N., Smith, C., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6021–6029.
Hoekstra, D., Wilschut, J., & Scherphoy, G. (1983) *Biochim. Biophys. Acta 732*, 327–332.
Houslay, M. D., & Stanley, K. K. (1982) *Dynamics of Biological Membranes*, Wiley, New York.
Kates, M. (1972) in *Techniques of Lipidology*, pp 360–361, North-Holland, Amsterdam.
Leonards, K. S., & Ohki, S. (1983) *Biochim. Biophys. Acta 728*, 383–393.
Leonards, K. S., & Ohki, S. (1984) *Biochemistry 23*, 2718–2725.
Majumdar, S., & Baker, R. F. (1980) *Exp. Cell Res. 126*, 175–182.
McIntyre, J. A., Gilula, N. B., & Karnovsky, M. J. (1974) *J. Cell Biol. 60*, 192–199.
Ohki, S., Düzgüneş, N., & Leonards, K. (1982) *Biochemistry 21*, 2127–2133.
Pal, R., Barenholz, Y., & Wagner, R. R. (1981) *Biochemistry 20*, 530–539.
Radin, N. A. (1969) *Methods Enzymol. 14*, 245–254.
Yeagle, P. L., Hutton, W. C., Huang, C.-H., & Martin, B. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3477–3481.
Zakai, N., Kulka, R. G., & Loyter, A. (1976) *Nature (London) 263*, 696–699.
Zakai, N., Kulka, R. G., & Loyter, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2417–2421.

Occurrence of Novel Antigenic Phosphoinositol-Containing Sphingolipids in the Pathogenic Yeast *Histoplasma capsulatum*†

Kathleen Barr and Robert L. Lester*

ABSTRACT: Five alkali-stable lipids from the yeast phase of *Histoplasma capsulatum* have been purified and analyzed. Each compound has equimolar amounts of hydroxysphinganine (phytosphingosine) and a hydroxy or nonhydroxy 24:0 fatty acid. All yield inositol phosphate after acid hydrolysis, and several are novel in that they also yield dimannosylinositol (compound V) and isomeric galactosyldimannosylinositols (compounds VI and VIII) after strong ammonolysis. The foregoing as well as other data suggest that compound V is a dimannosylinositolphosphoceramide and compounds VI and VIII are galactosyldimannosylinositolphosphoceramides with isomeric head groups. The chromatographic behavior of compounds II and III indicates that they are similar to the inositolphosphoceramides previously observed in *Saccharomyces cerevisiae*. Compounds V and VI are virtually absent from the mycelial phase of *H. capsulatum*. Antibodies that react with compounds V, VI, and VIII have been detected in sera from patients with histoplasmosis.

Sphingolipids have been implicated in the regulation of mammalian cell growth and communication. Some specific cell surface components, such as the ABH and Lewis blood group antigens, stage-specific embryonic antigens, and some tumor antigens, have been identified as glycosphingolipids (Hakomori, 1981).

Higher plants (Laine et al., 1980) and fungi (Brennan & Lösel, 1978) contain a group of sphingolipids, not found in animals, with a ceramide consisting of a long-chain base, usually hydroxysphinganine (phytosphingosine), N-acylated with hydroxy and nonhydroxy fatty acids and with polar head groups consisting of inositol, phosphate, and carbohydrate. Although no function has been ascribed to these charged molecules, the demonstration of a mutant strain of *Saccharomyces cerevisiae* with an absolute requirement of a long-chain base for growth and synthesis of sphingolipids suggests that one or more of these lipids plays a vital role (Wells & Lester, 1983).

Histoplasma capsulatum, the causative agent of histoplasmosis, is a pathogenic, dimorphic fungus. In the soil, *H. capsulatum* grows as a filamentous mycelium, but it converts to a yeast-like form in the tissues of infected animals. The mycelial and yeast phases can be maintained in the laboratory by incubation at 25 and 37 °C, respectively (Pine, 1954). The biochemical basis of morphogenesis is unknown, although observed phase transition related changes in cAMP concentrations (Maresca et al., 1977) and cell wall glycans (Domer et al., 1967; Domer, 1971) have implicated surface components in the transition.

The possible involvement of sphingolipids in phase-transition phenomena and the possibility that such lipids might evoke antibodies of diagnostic significance in histoplasmosis led us to purify five major alkali-stable phospholipids from the yeast phase of *H. capsulatum*. Two of these, designated as compounds II and III, are similar to the inositol phosphoceramides isolated from *S. cerevisiae* (Smith & Lester, 1974). Glyco-

† From the Department of Biochemistry, College of Medicine, University of Kentucky, Lexington, Kentucky 40536-0084. *Received March 12, 1984.* This research was supported in part by a grant from the Research Corporation. This work forms part of a dissertation to be submitted by K.B. to the University of Kentucky in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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sphingolipids V, VI, and VIII are novel compounds, containing mannopyranose, galactopyranose, and galactofuranose and a common oligosaccharide core, Man(α1→3)Man(α1→2 or 6)myoinositol (Barr et al., 1984). In this paper, we describe the purification and composition of these lipids as well as their reaction with antibodies from humans with histoplasmosis.

Experimental Procedures

Solvent Designations. Solvent A was $CHCl_3/CH_3OH/H_2O$ (16:16:5), adusted to pH 9.5 with concentrated NH_3; solvent B was $CHCl_3/CH_3OH$/concentrated NH_4OH/H_2O (50:36.8:6:7.2) plus 0.6 g/L NH_4Cl; solvent C was $CHCl_3/CH_3OH$/concentrated NH_4OH/H_2O (52.5:35.5:6:6) plus 0.6 g/L NH_4Cl.

Isolation and Purification of Alkali-Stable Lipids. A yeast-phase culture of the Illinois strain of *H. capsulatum* (obtained from Dr. Norman Goodman, University of Kentucky) was grown on brain heart infusion medium (Difco, 37 g/L) in 1.5-L amounts in 2-L flasks for 72 h with shaking at 37 °C. The yeast were killed by the addition of trichloroacetic acid to a final concentration of 5% and extracted according to Hanson and Lester's method IIIB (Hanson & Lester, 1980).

To destroy ester-containing phospholipids, the lipid extract resulting from a 100-L culture volume was treated with 1 volume of 0.2 N KOH in methanol for 60 min at room temperature followed by additions of 0.2 volume of 1.0 N acetic acid and 1.16 volumes of water. To separate nonlipid material from the alkali-stable lipids, the reaction mixture was added to a 200-mL Celite 545 (Fisher, washed with 1.0 N acetic acid until phosphorus free) column and washed with 1.2 L of a solution containing 0.06 M potassium acetate in ether/95% ethanol/CH_3OH/pyridine/H_2O (4.1:11.8:29.8:0.8:53.5) followed by 400 mL of CH_3OH/H_2O (1:1). The alkali-stable lipid fraction was eluted with 200 mL of methanol followed by 1.8 L of solvent A, dried in vacuo, and redissolved in 300 mL of H_2O/95% ethanol/ether/pyridine (15:15:5:1) and the entire transesterification procedure repeated again, yielding 513 μmol of P in the final alkali-stable lipid eluate.

Separation of the alkali-stable components was achieved by preparative liquid chromatography. Alkali-stable lipid (513 μmol of P) was dissolved in 90 mL of $CHCl_3/CH_3OH$/concentrated NH_4OH/H_2O (16:16:1:4), and 30-mL portions were chromatographed on a 2.5 cm × 200 cm column of Porasil (75/125 μm) maintained at 56 °C. Elution was initiated with a nonlinear gradient for 6 h, which was provided by a two-pump solvent delivery system (Model 6000A, Waters Associates) equipped with a Model 600 programmer (Waters Associates). The solvent composition changed from 0 to 20% solvent II, and the solvent composition at t min is given by % solvent II = $20(t/360)^{1/4}$. A linear gradient followed from 20 to 60% solvent II for 1.5 h. Solvent I was $CHCl_3/CH_3OH$/concentrated NH_4OH (65:29:6). Solvent II was $CHCl_3/CH_3OH$/concentrated NH_4OH/H_2O (40:42:6:12). Both solvents contained 0.6 g/L NH_4Cl. The flow rate was 9.9 mL/min, and fractions were collected every 3 min. Nonvolatile carbon in the eluate was monitored with a Pye Unicam moving-wire detector.

Pooled column fractions were dried in vacuo and dissolved in solvent A. The lipids were desalted by precipitation at 0 °C after the addition of 2 volumes of CH_3OH.

Purification of Compounds II and III. Pooled fractions II and III were dried and again subjected to the transesterification procedure described above. The alkali-stable lipid eluate from the Celite column was dried, redissolved in solvent A, and precipitated at 0 °C with the addition of 2 volumes of methanol. Compounds II (5.04 μmol of P) and III (22.5 μmol of P), dissolved in solvent A, were converted to the K^+ form by passage over a Dowex 50 (K^+) column equilibrated with the same solvent.

Purification of Compound VI. Further chromatography of compound VI was carried out on a 0.32 × 600 cm column of Porasil (37/75 μm) maintained at 56 °C by isocratic elution with solvent C. Six samples, each containing 3 μmol of phosphorus, dissolved in 0.5 mL of solvent A, were applied. Fractions were pooled on the basis of the moving-wire detector profile, and on the basis of this profile, compound VI was subdivided into compounds VIA (2.08 μmol of P) and VIB (1.76 μmol of P).

Analytical Chromatography of Purified Sphingolipids. Phospholipid samples, 0.2–0.5 μmol of P, were chromatographed isocratically on a 0.32 × 200 cm column of Porasil (37/75 μm) maintained at 56 °C. All solvents contained 0.6 g/L NH_4Cl and chloroform/95% ethanol/concentrated NH_4OH/water in proportions that varied with the compound examined: (compounds II and III) 43:46.8:6:4.2; (compound V) 40:48:6:6; (compounds VIA and VIB) 39:48.4:6:6.6; (compound VIII) 35:50:6:9. The eluates were monitored with the moving-wire detector. The isolated compounds were also chromatographed on silica gel plates (Whatman) with solvent C. The plates were sprayed with rhodamine and orcinol–H_2SO_4 (Skipski & Barclay, 1969).

Preparation of Labeled Sphingolipids. H. capsulatum was grown in 5 mL of brain heart infusion medium containing either 0.1 mCi of [^{3}H]inositol (New England Nuclear) or 5 mCi of [^{32}P]P_i (carrier free, New England Nuclear) for 2 days at 37 °C and for 4 days in either 5 mL of brain heart infusion medium containing 0.2 mCi [^{3}H]inositol or 15 mL of brain heart infusion medium that contained 15 mCi of [^{32}P]P_i. The cells were killed with trichloroacetic acid (TCA), lipid extracted, and transesterified twice, all as described for the large-scale preparation. The [^{3}H]inositol-labeled alkali-stable fraction was mixed with the ^{32}P-labeled fraction for chromatography on a 0.45 × 30 cm Lichrosorb Si60 column maintained at 56 °C. The programmed two-pump solvent delivery system described above provided the gradients for elution. Elution was initiated with the nonlinear gradient (no. 4) for 20 min, with the solvent composition varying from 0 to 35% solvent II; the solvent composition at t min can be described by % solvent II = $35(t/20)^{1/3}$. A linear gradient followed for 20 min as the solvent composition increased to 70% solvent II. Solvent I was $CHCl_3$/95% ethanol/concentrated NH_4OH (50:44:6). Solvent II was $CHCl_3$/95% ethanol/concentrated NH_4OH/H_2O (30:52:6:12). Both solvents contained 0.6 g/L NH_4Cl. The flow rate was 2.0 mL/min. Aliquots (20 μL) were taken from every 0.3-min fraction for scintillation spectrometry.

Alkali-Stable Phospholipids from Mycelial Phase of H. capsulatum. A mycelial phase culture of the Illinois strain of *H. capsulatum* (obtained from Dr. Norman Goodman) was grown for 11 days with shaking at 30 °C. The mycelia were killed by the addition of TCA and harvested by centrifugation, and the lipids were extracted and transesterified twice as described for the yeast phase culture.

The mycelial alkali-stable lipid eluate (3 μmol of lipid phosphorus) was chromatographed with the [^{3}H]inositol-labeled yeast-phase alkali-stable lipids (112000 cpm) on a 0.95 × 30 cm Lichrosorb Si60 column maintained at 56 °C. Elution was initiated with a nonlinear gradient (no. 4), which was provided as described above. During the first 45 min, the solvent composition changed from 0 to 20% solvent II, and the solvent composition at t min can be described by % solvent

II = $20(t/45)^{1/3}$. A linear gradient followed for 45 min as the solvent composition changed to 70% solvent II. Solvent I was $CHCl_3$/95% ethanol/concentrated NH_4OH (50:44:6) plus 0.6 g/L NH_4Cl. Solvent II contained $CHCl_3$/95% ethanol/concentrated NH_4OH/H_2O (30:52:6:12) plus 0.6 g/L NH_4Cl. The flow rate was 4 mL/min, and fractions were collected of which 10% was removed for scintillation spectrometry and the rest was assayed for total phosphorus.

Fatty Acid Analysis. The fatty acid composition of the lipids was determined by a combination of thin-layer and gas–liquid chromatography of methanolic HCl hydrolysates prepared by treating 0.2 μmol of each phospholipid in 1 mL of 3 N HCl in CH_3OH at 80 °C for 18 h. The samples were dried under a stream of nitrogen and redissolved in 0.2 mL of solvent A. Fatty acid methyl esters were analyzed on thin-layer plates with benzene/$CHCl_3$/acetic acid (90:10:1), and the esters were visualized with the rhodamine spray.

Quantitation and determination of the fatty acid chain length were done by gas chromatography with a Hewlett-Packard 5830A gas chromatograph equipped with a flame ionization detector in a 6 ft × 2 mm glass column packed with 3% OV 101 on 80/100 Supelcoport. The HCl methanolysates were dried with nitrogen and repeated 1-mL methanol additions and finally dried overnight in a desiccator. Trimethylsilyl esters were prepared (Sweeley et al., 1963), and arachidic acid (Eastman Organic Chemicals) was included in the methanolysis as an internal quantitative standard for compounds II, V, VIA, and VIII whereas stearic acid (Eastman Organic Chemicals) was used as an internal standard for compound III. Isothermal runs at 235 °C were employed for fatty acid identification while temperature programming from 190 to 235 °C at 5 °C/min was employed for quantitation. Fatty acid reference standards used for identification included 24:0 (lignoceric, Sigma), OH-24:0 (hydroxylignoceric, Applied Science Laboratories) and OH-18:0 (hydroxystearic, Calbiochem).

Long-Chain Base Analysis. After HCl methanolysis carried out as above, long-chain bases were resolved on silica gel thin-layer plates (Whatman) developed with $CHCl_3$/CH_3OH/2 N NH_4OH (40:10:1) and detected with ninhydrin. Reference standards (Sigma Chemical Co.) were D-4-hydroxysphinganine (phytosphingosine), DL-erythrosphinganine (dihydrosphingosine), and D-4-sphingenine (sphingosine). After HCl methanolysis and trimethylsilylation of 0.2-μmol lipid samples, the assignment of the long-chain base was confirmed by isothermal gas chromatography on a 6 ft × 2 mm glass column packed with 3% OV 101 with a Hewlett-Packard 5830A gas chromatograph at 235 °C with phytosphingosine as the reference standard. A modification (Smith & Lester, 1974) of the colorimetric method of Siakatos et al. (1971) was employed for quantitative determination with phytosphingosine as the standard.

Inositol Assay. Dried lipid samples, (0.1 μmol) were treated with 3 N HCl in CH_3OH for 18 h at 80 °C. The methanolysates were dried in a stream of nitrogen and redissolved in 1 mL of aqueous 6 N HCl and heated for 19 h at 120 °C. The hydrolyzed lipid was removed by three extractions with 0.3 mL of $CHCl_3$. The aqueous phase was taken to dryness and redried with absolute ethanol after addition of an internal standard, 0.1 μmol of mannitol. After acetylation (Albersheim et al., 1967), inositol hexaacetate was dissolved in 100 μL of acetone and analyzed by gas chromatography on a 6 ft × 2 mm glass column of 3% OV 225 on 80/100 Chromosorb W-H.P. at 200 °C.

Total Hexose and Carbohydrate Composition. Hexose was determined by the phenol–sulfuric method with mannose and galactose as standards (Dubois et al., 1966). Carbohydrate composition was measured by conversion of the sugars to their corresponding alditol acetates (Albersheim et al., 1967). The alditol acetates were dissolved in acetone for gas chromatographic analysis on a 6 ft × 2 mm glass column of 3% OV 275 on a 100/120 Chromosorb WAW at 210 °C with a Hewlett-Packard 5830A gas chromatograph.

Ammonolysis. To prepare the phosphorus-free intact glycosides from compounds V, VIA, and VIII, 5 μmol of P of V and VIII and 0.5 μmol of P of VIA were dried and treated with 1 mL of 10 N NH_4OH for 18 h at 150 °C. The volumes were increased to 3 mL with distilled H_2O before sequential extractions with 3 mL of diethyl ether and 3 mL of $CHCl_3$. The water phases were applied to 0.5-mL columns of AG 1X-2 (200–400 mesh) bicarbonate form and washed with 4 mL of H_2O. The oligosaccharide present after desalting was called the ammonolysate.

Acid Hydrolysis and Phosphatase Treatment of Radioactive Compounds III, V, VI, and VIII. Labeled compounds, purified by HPLC,[1] were hydrolyzed in 0.5 mL of aqueous 2 N HCl for 3 h at 100 °C. The volume was increased to 1.0 mL before three extractions with 0.5 mL of $CHCl_3$. The water phase was removed and taken to dryness several times under a stream of nitrogen, followed by additions of water to remove the HCl. The water-soluble products were examined by autoradiography of chromatograms on 589 orange ribbon paper (Schleicher & Schuell) developed with an ethanol/EDTA/NH_4HCO_3 solvent (Angus & Lester, 1972).

The hydrolysates were dissolved in 0.02 N ammonium acetate, pH 8.6, and incubated with 0.19 units of *Escherichia coli* alkaline phosphatase (Sigma) overnight at room temperature. Release of [^{3}H]inositol by phosphatase was detected by one-dimensional chromatography on 589 orange ribbon paper with the ethanol/EDTA/NH_4HCO_3 solvent. After autoradiography, the lanes were cut in 1-cm strips and counted by scintillation spectrometry.

Inositol Phosphate Chromatography. The solvent system used by Pizer & Ballou (1959) was employed for the analysis of inositol phosphate isomers obtained from acid hydrolysis of the radioactively labeled compounds. Aliquots of acid hydrolysates of the labeled compounds III, V, VI, and VIII were spotted on 589 orange ribbon paper. Inositol 2-phosphate (Sigma Chemical Co.) was used as a chromatographic standard. The chromatogram was developed for 42 h with 2-propanol/H_2O/concentrated NH_4OH (70:20:10). After autoradiography, the spots were cut out and counted for tritium and ^{32}P.

Phosphorus Analysis. Phosphorus was measured after perchloric acid digestion as described by Bartlett (1959).

Double Diffusion in Agar Gel. Agarose (Miles Laboratories) gels, 0.8% in phosphate-buffered saline (PBS, 0.0085 M Na_2HPO_4, 0.0017 NaH_2PO_4, 0.14 M NaCl), were prepared. *Histoplasma* lipids were dissolved in PBS with the aid of a sonic bath at a concentration of 0.12–0.18 μmol of lipid P/mL. The wells were filled with 25 μL of each of the following: *Histoplasma* compounds V, VIA, and VIII, mannosylbis(inositolphospho)ceramide from *S. cerevisiae*, histoplasmin (*Histoplasma* antigen, American Scientific Products), and sera from patients with histoplasmosis (provided by Dr. Norman Goodman). The gels were incubated overnight at

[1] Abbreviations: HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

room temperature in a humidifed chamber.

Enzyme-Linked Immunosorbent Assay (ELISA). To test the reaction of the purified *Histoplasma* lipids with antibodies in human sera, an ELISA test was developed (Blackburn & Schnaar, 1983; Hunter et al., 1982; Hunter & Brennan, 1983). Histoplasma compounds V, VIA, and VIII and *S. cerevisiae* lipids inositolphosphoceramide, mannosylinositolphosphoceramide, and mannosylbis(inositolphospho)ceramide were dissolved in 95% ethanol/H_2O (1:1) at a concentration of 17 nmol of lipid/mL. An aliquot (50 μL) of each was added to the wells of polyvinyl assay plates (Costar). The plates were left uncovered for 90 min at room temperature. After the solutions were removed, 100 μL of 1% bovine serum albumin (U.S. Biochemical Corp.) in PBS was applied, and the plates were incubated at 37 °C in a humid chamber for 1 h. The wells were washed 3 times with 100 μL of 0.1% Tween 85 in PBS, before addition of 50 μL of a 1/50 dilution of normal and patients' sera. All sera dilutions were made in 0.1% Tween 85 in PBS. Following an overnight incubation at 4 °C, the wells were washed 3 times with 100 μL of PBS. An aliquot (50 μL) of biotin-linked goat anti-human IgG (Vector Laboratories), diluted 1/500 in 0.1% Tween 85 in PBS, was added, and the plates were incubated for 1 h at room temperature. The wells were washed twice with 100 μL of PBS before addition of 50 μL of avidin D (Vector Laboratories), which had been diluted 1/50 in 0.1 M sodium bicarbonate. After the plates were incubated at room temperature for 30 min and then the wells were washed twice with 100 μL of PBS, 50 μL of biotinylated β-galactosidase (Vector Laboratories, diluted 1/500 in pH 7.5 buffer containing 10 mM Hepes, 1 mM $MgCl_2$, and 0.01% bovine serum albumin) was added to each well. The plates were incubated for 30 min at room temperature. The wells were washed 4 times with 100 μL of PBS followed by the addition of 100 μL of the substrate (5 mM *o*-nitrophenyl β-D-galactosidase in 0.05 M sodium phosphate, 5 mM KCl, 0.5 mM $MgSO_4$, and 50 mM 2-mercaptoethanol, pH 7.0). The plates were left at room temperature for 1 h, and the reaction was stopped by the addition of 10 μL of 1.0 M sodium carbonate. Absorbance was measured at 405 nm with a Micro Elisa reader (Dynatech Laboratories).

Results

Radioactively Labeled Alkali-Stable Lipids. The presence of novel, alkali-stable, phosphoinositol-containing lipids was detected in cells cultured with [^{32}P]P_i and [^{3}H]inositol for 2 and 4 days. Four major spots, evident in the thin-layer chromatographic pattern of the 4-day [^{32}P]P_i culture (Figure 1, lanes 1 and 6), were designated as compounds III, V, VI, and VIII. The 2-day profile was identical with the one obtained with lipids cultured for 4 days with [^{32}P]P_i. Additional minor compounds were evident in both ^{32}P samples and may represent variants of the major phospholipids.

Liquid chromatography of the alkali-stable lipids from *H. capsulatum* cultured for 2 days in medium containing [^{3}H]-inositol showed good resolution of four lipids (Figure 2) and, like thin-layer chromatography (Figure 1), exhibited additional minor peaks. This procedure was employed to isolate pure lipids from a mixture of alkali-stable lipids labeled for 4 days separately with [^{3}H]inositol and [^{32}P]P_i. Each peak was pooled and exhibited a single spot by thin-layer chromatography (Figure 1, lanes 2–5). These pooled samples were utilized for structural analysis (see below).

To find out if the major *Histoplasma* components were the same as one or more of the already characterized sphingolipids from *S. cerevisiae* (Smith & Lester, 1974), alkali-stable ^{32}P-labeled lipids from a semianaerobic culture of *S. cerevisiae*

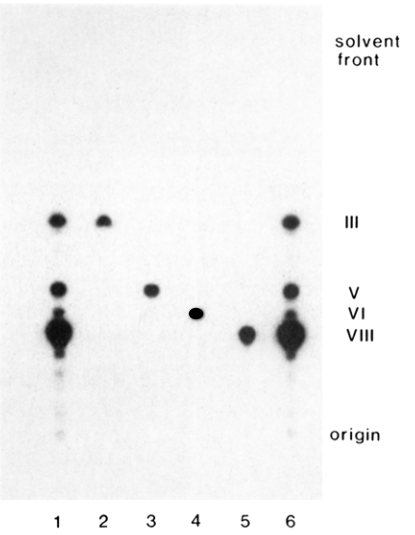

FIGURE 1: Thin-layer chromatography of [^{3}H]inositol-labeled ^{32}P-labeled alkali-stable lipids from *H. capsulatum*. As described under Experimental Procedures, [^{3}H]inositol-labeled ^{32}P-labeled, alkali-stable lipids were obtained from a 4-day culture of *H. capsulatum*. Silica gel thin-layer chromatography was carried out with solvent B (Experimental Procedures) followed by autoradiography. Total alkali-stable lipids were applied to lanes 1 and 6; compounds III, V, VI, and VIII, obtained by HPLC, were applied to lanes 2–5, respectively.

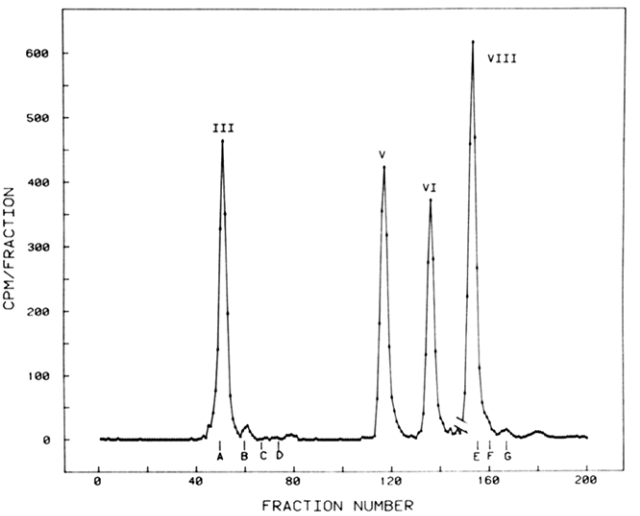

FIGURE 2: Liquid chromatography of [^{3}H]inositol-labeled alkali-stable lipids from *H. capsulatum* and ^{32}P-labeled lipids from *S. cerevisiae*. The alkali-stable ^{32}P-labeled lipids from a semianaerobic culture of *S. cerevisiae* strain MC6A were chromatographed with alkali-stable lipids from *H. capsulatum* cultured on [^{3}H]inositol on a 0.45 × 30 cm column of Lichrosorb Si60 (5 μm) maintained at 56 °C. Details of the gradient elution are included under Preparation of Labeled Sphingolipids. The tritium profile is denoted with solid trangles, and letters represent the location of the ^{32}P peaks. The latter were (A) inositol–P–ceramide (hydroxy fatty acid, phytosphingosine), (B) mannose–inositol–P–ceramide (non-hydroxy fatty acid, dihydrosphingosine), (C) mannose–inositol–P–ceramide (hydroxy fatty acid, dihydrosphingosine), (D) mannose–inositol–P–ceramide (hydroxy fatty acid, phytosphingosine), (E) mannose–(inositol–P)$_2$–ceramide (non-hydroxy fatty acid, dihydrosphingosine), (F) mannose–(inositol–P)$_2$–ceramide (hydroxy fatty acid, dihydrosphingosine), and (G) mannose–(inositol–P)$_2$–ceramide (hydroxy fatty acid, phytosphingosine). Counts per minute that appear after the break in the curve were divided by 3 before being plotted.

strain MC6A were chromatographed with the [^{3}H]inositol-labeled alkali-stable lipids from *H. capsulatum*; the letters in Figure 2 indicate the retention times of the *Saccharomyces* lipids. Only *H. capsulatum* compound III appeared to be one of the *S. cerevisiae* sphingolipids. However, it was reproducibly

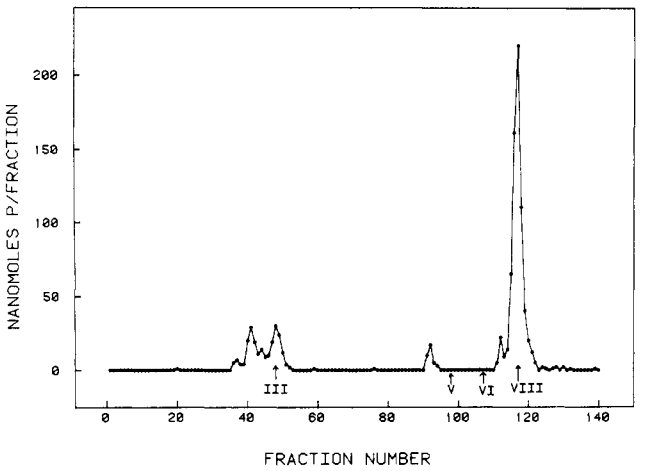

FIGURE 3: Comparison of the alkali-stable lipids from the yeast and mycelial phase. Alkali-stable lipids from the mycelial phase were chromatographed with the yeast-phase [^{3}H]inositol-labeled alkali-stable lipids on a 0.95 × 30 cm column of Lichrosorb Si60 (5 μm) (Experimental Procedures). Total phosphorus of the mycelial lipids is denoted with solid circles, and Roman numerals indicate the peak retention times of the four major yeast-phase lipids (Figure 2).

very slightly more retained than the inositol phosphoceramide from *S. cerevisiae* (Figure 2A), whose ceramide consists of a C_{26} hydroxy fatty acid and phytosphingosine, whereas the compound from *H. capsulatum* contains C_{18} and C_{24} hydroxy fatty acids (see below). Compounds V and VI were significantly more polar than the *S. cerevisiae* mannosylinositol-phosphoceramides (B–D), thus indicating the uniqueness of these *Histoplasma* compounds. Peak VIII from *Histoplasma* was retained less than the least polar variant of mannosyl-bis(inositolphospho)ceramide (E, Figure 2), whose ceramide consists of a non-hydroxy fatty acid and dihydrosphingosine. Thus, compounds V, VI, and VIII are not represented in *S. cerevisiae* and appear to be novel compounds.

Unlabeled alkali-stable lipids from the mycelial phase were chromatographed on a larger column with marker [^{3}H]-inositol-labeled alkali-stable lipids of the yeast phase (Figure 3). The chromatographic pattern obtained from the mycelial lipids, detected as chemical phosphorus, showed a striking absence of compounds V and VI. Compounds III and VIII appear to be present in both phases, with compound VIII as the most abundant alkali-stable lipid.

Isolation of Yeast-Phase Alkali-Stable Lipids. Further chemical characterization of these novel lipids necessitated a large-scale isolation. Therefore, yeast-phase *H. capsulatum* was cultured in 100 L of medium, and the lipids were extracted and transesterified. Analysis of a small sample of the total lipid extract treated to remove nonlipid phosphorus showed that, prior to transesterification, there were 50.9 μmol of lipid phosphorus/g dry wt of cells of which 10% remained after alkaline methanolysis. Although the resolution was not as good as that seen in Figure 1 with a high-performance support, preparative liquid chromatography of the mild alkali stable lipid mixture did show six major peaks in the nonvolatile carbon moving-wire detector profile (Figure 4). Before being pooled aliquots of peak fractions were chromatographed on silica gel plates (Whatman) with solvent C. The lipids of peaks I–III were only rhodamine-positive but the material from peaks V, VI, and VIII were glycolipids as judged by positive reactions to both orcinol–H_2SO_4 (Skipski & Barclay, 1969) and rhodamine. Each peak contained phosphorus, and all the phosphorus applied to the column was recovered, including some found in the nonsorbed region and in some minor components. Material from peaks III, V, VI, and VIII comigrated on

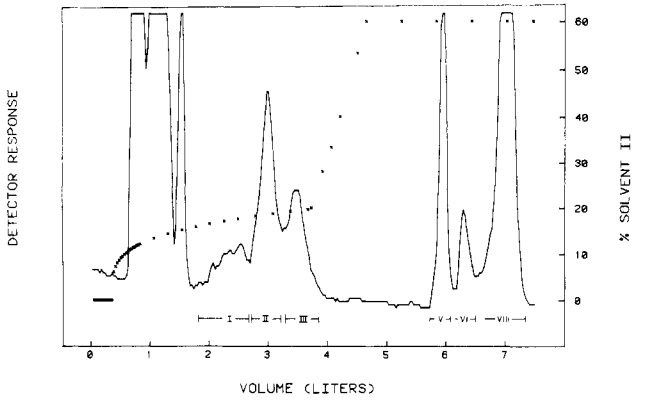

FIGURE 4: Preparative liquid chromatography of the alkali-stable lipids. Alkali-stable lipid was chromatographed on a 2.5 × 200 cm silica gel column as described under Experimental Procedures. The solid lines denote the relative carbon detector response, and (×) depicts the gradient composition. Peaks were pooled as indicated by brackets.

thin-layer plates with the HPLC-purified radioactively labeled compounds (Figure 1, lanes 2–5). Thin-layer chromatography of samples (25 nmol) of the pooled and precipitated material of peaks V (30 μmol of P) and VIII (92 μmol of P) indicated that both lipids were free of contamination. These two alkali-stable lipids were designated compounds V and VIII, respectively, and were not subjected to any further purification.

Purification of Compounds II and III. Unlike V and VIII, peaks I–III were contaminated with multiple rhodamine-positive spots, believed to be acyl ester lipid. Therefore, each peak was again subjected to the transesterification procedure. All of the phosphorus of peak I was isolated in the nonlipid fraction after partitioning on Celite and was not examined further. After the second round of transesterification, 54% of the phosphorus of peak II and 75% of that of peak III were recovered from Celite in the alkali-stable lipid eluate. The material that remained stable to alkaline methanolysis was denoted compounds II and III. Compound II was never observed in the small-scale radioactive experiments (Figures 1 and 2) but was present in duplicate large-scale culture experiments.

Purification of Compound VI. The pooled fractions corresponding to peak VI (Figure 4) exhibited a contaminating spot sensitive to rhodamine and orcinol–H_2SO_4 with an R_f similar to compound V after the silica gel thin-layer chromatography (Experimental Procedures). Therefore, several portions of VI were chromatographed isocratically (Experimental Procedures). What had appeared by thin layer to be compound V was eluted sooner than its expected retention volume in the liquid chromatogram. Following this were two, not well resolved, glycolipid peaks in the region expected for the major component. The peaks were pooled, precipitated, and designated as compounds VIA (shorter retention time) and VIB. Neither chromatographed on thin-layer plates with compound V, and each gave single spots that could not be separated from each other.

Assessment of Purity of Isolated Lipids by Isocratic Liquid Chromatography. Each lipid was judged to be pure on the basis of thin-layer chromatography, with the rhodamine and orcinol–H_2SO_4 reagents. Further confirmation of purity was obtained from liquid chromatography. Each compound was chromatographed isocratically (Experimental Procedures) with elution monitored with the carbon detector (Figure 5). In both thin-layer and liquid chromatography systems, compound II migrated as a single moiety. Although judged to be pure on silica gel plates, the liquid chromatography profile of compound III included a shoulder with the retention time of

Table I: Identification of Fatty Acids and Long-Chain Bases by Analysis of Methanolic Hydrolysates

	retention time of standard (min)	retention times found for compound[a]				
		II	III	V	VIA	VIII
hydroxysphinganine	6.10	6.10	6.09	6.10	N/A	6.10
fatty acids[b]						
OH-18:0	2.65		2.65 (16)			
OH-22:0	7.62			7.62 (8)	N/A (9)[c]	
24:0	7.62	7.62 (100)	7.59 (17)			
OH-24:0	13.27		13.27 (67)	13.27 (92)	N/A (91)[c]	13.27 (100)

[a] Aliquots of each lipid were treated with the silylation reagent after HCl methanolysis and subjected to gas chromatography as described under Experimental Procedures. Retention time is in min. [b] Values in parentheses are percent FID response. [c] From temperature-programmed analysis.

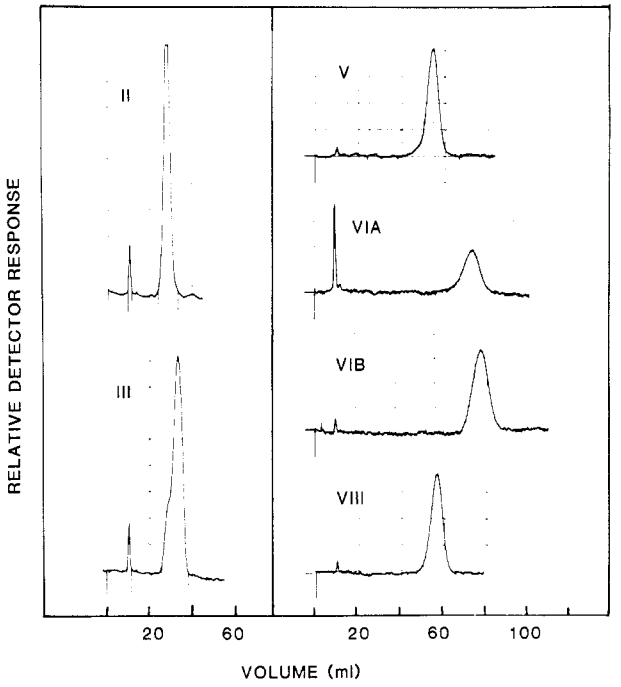

FIGURE 5: Isocratic liquid chromatography of purified compounds. Aliquots of each compound were chromatographed on a 0.32 × 200 cm silica gel column as described under Experimental Procedures. The eluates were monitored with the moving-wire detector.

compound II. On the basis of the detector response, this shoulder represented less than a 10% comtamination with compound II. Since compounds II and III were similar (see below) to those isolated from *S. cerevisiae* (Smith & Lester, 1974) and did not represent novel compounds, there was no attempt to further purify compound III. Liquid chromatography of compounds V and VIII confirmed that each represented a pure alkali-stable phospholipid. Compounds VIA and VIB exhibited overlapping profiles under the isocratic conditions used. When monitored by carbon detection, a nonsorbed peak was observed in the profile of compound VIA. This peak was not detected by either the rhodamine or the orcinol–H_2SO_4 reagents by thin-layer chromatography. Since this non-lipid, non-carbohydrate contaminant represented less than 10% of the carbon detector response of VIA, this compound was not further purified. Further characterization was carried out with compound VIA only.

Fatty Acid, Long-Chain Base, and Inositol Analysis. Qualitative analysis of compounds II, III, V, VIA, and VIII was based on thin-layer chromatography of HCl methanolysates. All of the compounds yielded a strong ninhydrin-positive spot that comigrated with standard phytosphingosine. Additionally, compounds II and III also contained a faint spot with the retention time of standard DL-erythrodihydrosphingosine. With a solvent system that separates the methyl esters of nonhydroxylated fatty acids from mono- and dihydroxy fatty acids (Experimental Procedures) and detection with rhodamine, compound II exhibited only non-hydroxy fatty acids whereas only monohydroxy fatty acid methyl esters were evident in the methanolysates of the other four compounds.

Table II: Summary of Quantitative Analyses

	II	III	V	VIA	VIII
Intact Sphingolipids (mol/mol of P)					
long-chain base	0.84	1.07	1.06	0.83	1.13
total fatty acid[a]	1.12	1.13	1.04	1.01	1.09
inositol	1.01	1.09	1.19	0.98	1.04
total carbohydrate[b]	0.09	0.07	2.14	3.24	3.35
mannose	ND[c]	ND	2.2	2.15	1.95
galactose	ND	ND	0.0	1.05	0.98
Ammonolysis Products (mol/mol of Inositol)					
inositol	ND	ND	1.0	1.0	1.0
mannose	ND	ND	1.8	2.1	2.1
galactose	ND	ND	0.0	1.0	1.01

[a] The total fatty acid is the sum of those fatty acids indicated in Table I. [b] Mannose was used as a standard in the phenol–sulfuric analysis of compounds II, III, and V. Mannose and galactose in a ratio of 2:1 were the standards for carbohydrate analysis of compounds VIA and VIII. [c] ND, not determined.

The fatty acids were quantified and identified by gas–liquid chromatography of the trimethylsilyl esters of the HCl methanolysates (Table I), each compound exhibiting 1 mol of fatty acid/mol of phosphorus (Tables I and II).

The long-chain base was quantified colorimetrically and gave a long-chain base to phosphorus ratio of 1:1 (Table II) for all the compounds. C_{18}–phytosphingosine was also identified as the long-chain base constituent when the silylated HCl methanolysates were examined by gas–liquid chromatography (Table I). Each compound contained 1 mol of inositol/mol of phosphorus (Table II).

The presence of 1 mol of fatty acid, long-chain base, and inositol per phosphorus indicated that these alkali-stable lipids were phosphoinositol sphingolipids. All of the compounds had the same long-chain base, phytosphingosine; compound II had a nonhydroxylated 24:0 fatty acid in the ceramide moiety, whereas the others contained mostly hydroxylated 24:0. Compounds V, VI, and VIII were orcinol–H_2SO_4 positive, suggesting that differences in carbohydrate composition were responsible for the chromatographic separation of these compounds.

Carbohydrate Analysis. When assayed by the phenol–sulfuric method, compound V gave 2 equiv of hexose/mol of phosphorus (Table II). Although separable by thin-layer and liquid chromatography, both compounds VIA and VIII had 3 hexose equiv. No hexose was found associated with either compound II or compound III (Table II). Analysis by gas–liquid chromatography of the carbohydrate moieties after conversion to alditol acetates demonstrated that compounds VIA and VIII had the identical hexose composition: two mannoses and one galactose. Compound V contained 2 equiv of mannose (Table II).

Treatment of sphingolipids with 10 N NH_4OH overnight at 150 °C cleaves phosphomonoester and -diester bonds but does not affect glycosidic linkages (Ballou et al., 1963). Ammonolysis of compounds V, VIA, and VIII produced single, unique oligosaccharides detected with the orcinol–H_2SO_4 reagent that could be separated on silica gel thin-layer plates with double development with acetonitrile/H_2O (2:1). The relative R_f values were (V) 0.39, (VIA) 0.33, and (VIII) 0.26. The ammonolysates of compounds V and VIII were acetylated with acetic anhydride/pyridine (1:1) for 2 h at 100 °C. No free inositol hexaacetate was found associated with the ammonolysates of compounds V and VIII when the acetylated preparations were analyzed by thin-layer and gas–liquid chromatography. We conclude that the inositol and all of the hexose of each compound are linked. Conversion of these oligosaccharides to alditol acetates showed that V consisted of two mannose molecules and one inositol (Table II). Alditol acetate analysis of VIA and VIII revealed no compositional differences between these compounds. Both contained two mol of mannose, 1 mol of galactose, and 1 mol of inositol (Table II).

Liberation of Inositol Phosphate from All Sphingolipids. Inositol phosphate was obtained from aqueous 2 N HCl hydrolysis (2 h, 100 °C) of [^{3}H]-inositol-labeled ^{32}P-labeled III, V, VI, and VIII. After hydrolysis, 90% of the radioactivity was recovered in the water-soluble fraction. The appearance of free inositol before and after phosphatase digestion of the HCl hydrolysate was monitored by paper chromatography (Experimental Procedures). Prior to enzymatic digestion, 80–85% of the ^{3}H migrated with the R_f of inositol monophosphate, and the remainder migrated as free inositol. All of the ^{32}P chromatographed with the R_f of inositol monophosphate and P_i, which were not separated by this system. Digestion of the water-soluble products with *E. coli* alkaline phosphatase converted all of the putative inositol phosphate to free inositol. These results demonstrated that the phosphate is linked to the inositol in all four intact sphingolipids.

Stability of Labeled Sphingolipids to Alkali. Compositionally, compound III is very similar to the *S. cerevisiae* inositolphosphoceramides; these were shown to be labile to alkali (Smith & Lester, 1974). On the other hand, the mannosylinositolphosphoceramides from *S. cerevisiae* were stable to such alkaline treatment (Smith & Lester, 1974), probably due to mannosylation vicinal to the phosphate group, preventing facile formation of a cyclic inositol phosphate. Therefore, the stability of the labeled *H. capsulatum* sphingolipids to alkali was examined. Aliquots of ^{3}H-labeled ^{32}P-labeled lipids were treated with 1 N KOH at 37 °C overnight. The reaction mixture was neutralized with glacial acetic acid and examined by chromatography on 589 orange ribbon paper with $CHCl_3$/CH_3OH/4.2 N NH_4OH (9:7:2), followed by autoradiography. Only compound III was alkali labile since all of the ^{32}P and [^{3}H]inositol remained at the origin as expected for an inositol monophosphate. After this alkali treatment, V, VI, and VIII migrated as intact lipids. The product of alkaline hydrolysis of compound III was completely susceptible to *E. coli* alkaline phosphatase, yielding free inositol as monitored by paper chromatography (see above). It is therefore concluded that compound III is an inositolphosphoceramide with a structure similar to the *S. cerevisiae* compound and that the phosphoinositol moieities of compounds V, VI, and VIII are substituted vicinal to the phosphate group.

Linkage of Phosphate and Inositol. On the basis of its infrared spectrum, chromatographic properties, and optical activity (Pizer & Ballou, 1959), it was established that inositol 1-phosphate is the major inositol monophosphate resulting from alkaline degradation of soybean phosphatidylinositol, and therefore, the phosphate is considered to be linked to the 1-position of inositol in phosphatidylinositol. A limited amount of material precluded a similar approach with the *Histoplasma* lipids as well as the fact that compounds V, VI, and VIII are resistant to alkaline hydrolysis. Although it is difficult to establish with certainty the linkage of phosphate to inositol in complex lipids by acid hydrolysis due to acid-catalyzed migration of the inositol phosphate esters (Pizer & Ballou, 1959), we thought it would be of some value to compare the set of major inositol phosphate isomers resulting from the acid-catalyzed hydrolysis of phosphatidyl[^{3}H]inositol and the labeled *Histoplasma* compounds. Using descending paper chromatography (Pizer & Ballou, 1959), we found that each compound gave the same set of isomeric inositol monophosphates. These products were identical with those obtained from acid-catalyzed hydrolysis of phosphatidyl[^{3}H]inositol from *S. cerevisiae*. We conclude that the phosphoinositol linkage is the same for all these compounds, most likely at the 1-position. Because of the formation of isomeric inositol phosphates, further work is necessary for unequivocal proof.

Table III: Detection of Antibodies to *Histoplasma* Sphingolipids in Humans with Histoplasmosis

	absorbance at 405 nm (×10^3) for sphingolipid[b]					
	V		VIA		VIII	
sera[a]	−Ag	−AntiAb	−Ag	−AntiAb	−Ag	−AntiAb
132	69	144	221	296	189	156
449	238	307	270	243	594	660
399	193	275	204	295	305	392
645	115	196	1427	1509	444	507
552	10	0	201	206	32	5
A	15	23	16	30	24	35
B	15	23	49	62	−5	6
C	33	84	−16	38	99	159
D	8	17	17	30	1	14

[a] Control sera are designated by letters, and numbers represent patients' sera. [b] The absorbance values (405 nm) represent the average of three determinations. In the first lane (−Ag), the absorbance of the well without the sphingolipid has been subtracted, and the second lane (−AntiAb) includes the subtraction of the absorbance of the well without anti-immunoglobulin.

Reaction of Compounds V, VI, and VIII with Antibodies. Since complex glycosphingolipids are known to be antigenic in animals, it was of interest to examine sera for antibodies directed at the novel lipids purified from *H. capsulatum* in those individuals suspected of histoplasmosis whose sera showed positive titer against histoplasmin in the standard complement fixation assay. Our first approach was to examine the interaction of the *Histoplasma* lipids with sera from these individuals by double immunodiffusion in agarose gels. Compound VI formed a precipitin band with 1 of 11 different patients' sera tested in this manner; compounds V and VIII did not react with any of the sera. The inclusion of exogenous lipids (Graf & Rapport, 1965), such as phosphatidylcholine, did not improve the sensitivity. In contrast, an enzyme-linked immunosorbent assay (ELISA) gave results (Table III) showing that each patient's serum reacted significantly better with one or more of the purified sphingolipids than did the four randomly selected human control sera. Compound VI was reactive with all of the patient's sera, particularly serum 645. The reactivities of compounds V and VIII were more variable. Neither lipid reacted with serum 552, but compound VIII was more reactive than VI with serum 449. The absorbance values of the control sera were negligible when compared with those obtained from patient's sera. Sphingo-

lipids from *S. cerevisiae*, inositolphosphoceramide, mannosylinositolphosphoceramide, and mannosylbis(inositolphospho)ceramide (Smith & Lester, 1974) did not react in a comparable ELISA experiment (not shown), indicating a specificity of the reaction between the *Histoplasma* lipids and patient's sera.

Discussion

In summary, we have isolated and characterized five pure phosphoinositol sphingolipids from the yeast phase of *H. capsulatum* that are related to previously characterized fungal and plant phosphoinositolceramides. Two of these, compounds II and III, are variants of the inositolphosphoceramides isolated from *S. cerevisiae* (Smith & Lester, 1974). Compounds V, VIA, and VIII are novel compounds. On the basis of the work presented in this paper, the structural information can be summarized as follows: compound II, ceramide–P–inositol; compound III, OH–ceramide–P–inositol; compound V, OH–ceramide–P–inositol–[mannose$_2$]; compound VIA, OH–ceramide–P–inositol–[mannose$_2$, galactose]; compound VIII, OH–ceramide–P–inositol–[mannose$_2$, galactose].

The linkages, anomeric configurations, and sequences of the carbohydrate moieties of compounds V, VI, and VIII are reported in the following paper (Barr et al., 1984). Although well separated by thin-layer and liquid chromatography, compounds VIA and VIII are structural and compositional isomers (Barr et al., 1984). Compound VIII, or a compound resembling this molecule, is found in both the mycelial and yeast phases. Compounds V and VI appear to be restricted to the yeast phase.

The observation that compounds such as V, VI, and VIII react with antibodies from histoplasmosis patients is not surprising since many antigens have been identified as glycosphingolipids (Hakomori, 1981). However, it cannot be ruled out that the true immunogen in humans might have been another glycoconjugate, such as a glycoprotein, which could have saccharide structures similar to the glycolipids. Clinical diagnosis of histoplasmosis can be established by two different serological methods: complement fixation tests that use either whole yeast-phase cells or mycelial histoplasmin as antigens and agar double-immunodiffusion analysis with histoplasmin (Bradley et al., 1974). Histoplasmin is a preparation obtained from filtrates of liquid culture media in which *H. capsulatum* has been grown at 25 °C for varying lengths of time, the activity of which may reside in one or more glycoproteins (Bradley et al., 1974).

Well-defined low molecular weight compounds, such as compounds V, VI, and VIII, which have been isolated from live cells, may offer some diagnostic advantages, especially if one or more of the *Histoplasma* compounds is not associated with other pathogenic fungi. The fact that these compounds are phase specific increases the attractiveness of their application to the clinical diagnosis of histoplasmosis.

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References

Albersheim, P., Nevins, D., English, P., & Karr, A. (1967) *Carbohydr. Res. 5*, 340–345.

Angus, W., & Lester, R. L. (1972) *Arch. Biochem. Biophys. 151*, 483–495.

Ballou, C. E., Vilkas, E., & Lederer, E. (1963) *J. Biol. Chem. 238*, 69–76.

Barr, K., Laine, R. A., & Lester, R. L. (1984) *Biochemistry* (following paper in this issue).

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Blackburn, C. C., & Schnaar, R. L. (1983) *J. Biol. Chem. 258*, 1180–1188.

Bradley, G., Pine, L., Reeves, M. W., & Moss, C. W. (1974) *Infect. Immun. 9*, 870–880.

Brennan, P. J., & Losël, D. M. (1978) *Adv. Microb. Physiol. 17*, 47–179.

Domer, J. E. (1971) *J. Bacteriol. 107*, 870–877.

Domer, J. E., Hamilton, J. G., & Harkin, J. C. (1967) *J. Bacteriol. 94*, 466–474.

Dubois, M., Giles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.

Graf, L., & Rapport, M. M. (1965) *Int. Arch. Allergy Appl. Immunol. 28*, 171–177.

Hakomori, S. (1981) *Annu. Rev. Biochem. 50*, 733–764.

Hanson, B. A., & Lester, R. L. (1980) *J. Lipid Res. 21*, 309–315.

Hunter, S. W., & Brennan, P. J. (1983) *J. Biol. Chem. 258*, 7556–7562.

Hunter, S. W., Fujiwara, T., & Brennan, P. (1982) *J. Biol. Chem. 257*, 15072–15078.

Laine, R. A., Hsieh, T. C.-Y., & Lester, R. L. (1980) *ACS Symp. Ser. No. 128*, 65–78.

Maresca, B., Medoff, G., Schlessinger, D., Kobayashi, G. S., & Medoff, J. (1977) *Nature (London) 266*, 447–448.

Pine, L. (1954) *J. Bacteriol. 68*, 671–679.

Pizer, F. L., & Ballou, C. E. (1959) *J. Am. Chem. Soc. 81*, 915–921.

Siakatos, A. N., Kulkarni, S., & Passo, S. (1971) *Lipids 6*, 254–259.

Skipski, V. P., & Barclay, M. (1969) *Methods Enzymol. 14*, 545–546.

Smith, S., & Lester, R. L. (1974) *J. Biol. Chem. 249*, 3395–3405.

Sweeley, C. C., Bentley, R., Makita, M., & Wells, W. W. (1963) *J. Am. Chem. Soc. 85*, 2497–2507.

Wells, G. B., & Lester, R. L. (1983) *J. Biol. Chem. 258*, 10200–10203.