Characterizations of *Pneumocystis carinii* and rat lung lipids: glyceryl ethers and fatty alcohols

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Abstract ***Pneumocystis carinii carinii*** **and rat lung phospholipids contained 3–6% 1-alkyl-2-acyl glycerols composed of the glyceryl ether species, 1-O-octadecyl glycerol (batyl alcohol), 1-O-octadec-9-enyl glycerol (selachyl alcohol), 1-O-hexadecyl glycerol (chimyl alcohol), and 1-O-hexadec-9-enyl glycerol. Of the major phospholipid classes, phosphatidylinositol (PI) and phosphatidylserine contained the highest percentage of alkyl acyl glycerols. Methylprednisolone treatment caused an increase in alkyl acyl PI of rat lung lipids from 12% to 45%. As the PI concentration in lung phospholipids increases in rats treated with methylprednisolone, the increase in alkyl acyl PI was substantial; the proportions of alkyl acyl phosphatidylethanolamine and alkyl acyl lyso phosphatidylcholine (PC) also increased.** ***Pneumocystis*** **phospholipids contained higher proportions of alkyl acyl PC than the phospholipids of the lungs from normal and immunosuppressed uninfected rats. The glyceryl ether compositions of** ***P. carinii carinii*** **PC and lyso PC were similar, which suggests that lyso PC in the organism is derived by phospholipase A_2 action on PC. This was not the case for PC and lyso PC of the lung controls. Analysis of the free fatty alcohols, precursors of glyceryl ethers identified only saturated species in** ***P. carinii carinii*** **and rat lung controls. Thus, the introduction of a double bond in the alcohol moiety of glyceryl ethers occurs after formation of the ether linkage between fatty alcohol and the glyceryl backbone.—** Kaneshiro, E. S., Z. Guo, D. Sul, K. A. Kallam, K. Jayasimhulu, and D. H. Beach. **Characterization of** ***Pneumocystis carinii*** **and rat lung lipids: glyceryl ethers and fatty alcohols.** ***J. Lipid. Res.*** **1998.** 39: **1907–1917.**

Supplementary key words AIDS • batyl alcohol • chimyl alcohol • long chain alcohols • 1-O-hexadec-9-enyl glycerol • 1-O-octadec-9-enyl glycerol • phospholipids

Pneumocystis carinii pneumonia (PcP) remains a serious infectious disease among immunocompromised individuals such as AIDS patients. The lack of a continuous axenic culture method has slowed progress in understanding the biochemical nature of this important opportunistic organism. However, recent advances have been developed for producing highly purified *P. carinii* preparations isolated from infected mammalian lungs (1, 2). The purity of organisms prepared by methods developed in our laboratory was rigorously demonstrated (1). Using these purification protocols, credible biochemical studies on this organism are now feasible. Several lipid biochemical studies have been performed using this organism preparation (3–9).

Glyceryl ethers include those compounds that have fatty alcohols linked to glycerol backbones via C–O–C bonds (alkyl glycerolipids). Ether bonds are much more stable than ester bonds by which fatty acids are linked to glycerolipids (10, 11). Ether lipids are widely distributed, occurring in organisms from bacteria to mammals (10, 11). Current interest in ether lipids has been stimulated by the finding that choline glyceryl ethers are potent bioactive compounds known as platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (12–14). Instillation of PAF into the lungs of experimental animals has been shown to cause acute pulmonary inflammation (15), and type II pneumocytes increase surfactant secretion in response to PAF (16, 17). In bacterial pneumonia, PAF released by host cells in response to the infection, has been implicated as a pathogenic mechanism; the administration of a PAF antagonist increased survival of experimental animals (18). While ether lipids are normal components of lung tissue, the structures of these compounds have not been characterized in detail in the rat, and it is

Abbreviations: FAB, fast atom bombardment; FID, flame ionization detector; GLC, gas–liquid chromatography; LSI, liquid secondary ion; MeOH, methanol; MS, mass spectrometry; MOPS, 3-(N-morpholino)propane sulfonic acid; NMR, nuclear magnetic resonance; PAF, platelet-activating factor; PC, phosphatidylcholine; PcP, *Pneumocystis carinii* pneumonia; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; P_i, inorganic phosphate; PS, phosphatidylserine; RRT, relative retention time; TLC, thin-layer chromatography; TMS, trimethylsilyl.

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not known whether corticosteroid immunosuppression used in the PcP rat model alters the content or composition of glyceryl ethers. In the present study, we analyzed the fatty alcohols and glyceryl ethers of *P. carinii carinii* and compared them with those of lungs from normal rats and immunosuppressed uninfected rats.

MATERIALS AND METHODS

Organisms

Mixed life cycle stages of *Pneumocystis carinii carinii* were obtained from the Boylan and Current (19) infected rat model as previously described (1). Animals that were immunosuppressed but not inoculated were used as controls (immunosuppressed uninfected rats). Rats that were not immunosuppressed nor infected also served as controls (normal rats). All control animals were placed under double-barrier conditions (1, 19).

Pneumocystis organisms were isolated from the infected rat lungs and purified by protocols previously detailed (1). Briefly, the lungs were first perfused to eliminate blood elements, then excised. After being cut into small pieces, the lung tissue samples were subjected to homogenization with a Stomacher apparatus (Tekmar, Inc., Cincinnati, OH) in the presence of the mucolytic agent, glutathione. The homogenate was then placed in a sieve, and the sievate was subjected to a series of low- and high-speed centrifugation steps to eliminate lung surfactant and other contaminants. The organism preparation was finally filtered through tandemly arranged Sweeney 8-μm-pore and 5-μm-pore microfilter syringe adaptor units.

To prepare lung controls for lipid extraction, the lungs were perfused and cut into pieces and processed in the Stomacher apparatus. The resultant homogenate was then disrupted by passage through a French pressure cell (Aminco, Urbana, IL) at 20,000 psi.

Lipid extraction and isolation of purified lipid classes

All organic solvents used in this study were redistilled and contained butylated hydroxytoluene as an anti-oxidant (20) except in solvents used to dissolve lipids for final analytical procedures, e.g., gas–liquid chromatography (GLC). The lung homogenates of normal and immunosuppressed uninfected rat controls, and the purified *P. carinii* preparations were extracted for total lipids using a neutral monophasic solvent system with chloroform ($CHCl_3$)–methanol (MeOH)–biological sample in the proportions of 1:2:0.8 (v/v/v) (21). After extraction, lipid purification was achieved by adding $CHCl_3$ and 0.29% aqueous NaCl to form a biphasic system (final proportions were $CHCl_3$–MeOH–H_2O 2:1:0.6 (v/v/v)) (22). The insoluble solid cellular material was collected at the interphase. The lower organic phase was recovered and its volume was reduced by rotary evaporation (Rotovap, Brinkmann Instruments, Westbury, NY) and dried under a stream of N_2 (N-Evap, Organomation, Berlin, MA).

Total phospholipids were isolated by adsorption column chromatography using silicic acid (Unisil, Clarkson Chemical Co., Williamsport, PA). The neutral lipid fraction was first removed by elution with 30–50 ml of $CHCl_3$ then the total polar lipid fraction was recovered by elution with 30–50 ml of MeOH.

Thin-layer chromatography (TLC)

Analytical TLC was done on glass-backed 250-μm thick Silica G plates (Whatman, Kent, U.K.). After development in $CHCl_3$–MeOH–acetic acid–H_2O 50:25:7:3, (by volume; SS #1), the plates were sprayed for visualization of phospholipids according to Vaskovsky and Kostetsky (23). Choline phospholipids were identified by staining with the Dragendorff reagent (20). Phospholipid compositions were determined by quantitation of phosphate-positive spots on TLC plates by densitometry on a Shimadzu CS9000U dual-wavelength flying spot scanner (Shimadzu Scientific Instruments, Columbia, MD) at 650 nm.

Individual phospholipid classes were separated and purified from the polar lipid fraction by preparative TLC using glass-backed plates coated with 500-μm thickness of Silica Gel G (Alltech, Deerfield, IL) (**Fig. 1**). The plates were first developed using SS #1, and then lipid bands were visualized with I_2 vapor. Cardiolipin, phosphatidylcholine (PC), sphingomyelin, lyso PC, and other minor bands were well resolved. These lipid bands were individually scraped, and the lipids were eluted from the Silica Gel with $CHCl_3$–MeOH 1:2 (v/v), then dried under N_2. Phosphatidylinositol (PI) was not completely separated from phosphatidylserine (PS) and phosphatidylethanolamine (PE) was not completely separated from phosphatidylglycerol (PG). The two bands containing these two sets of unresolved lipids were individually scraped, and the lipids were eluted, dried, and then subjected to TLC under different conditions. Using SS #1, PI and PS were separated by using aluminum-backed plates coated with 250 μm Silica Gel. Similarly, PE and PG were resolved on aluminum-backed plates; however, in this case, $CHCl_3$–MeOH–H_2O 65:25:4 (v/v/v; SS # 2) was used as the solvent system.

Sequential chemical degradation of total phospholipids

To determine the distribution of *P. carinii* phospholipid subclasses according to the presence of different types of chemical bonds (diacyl, acyl-alkenyl, acyl-alkyl, or ceramide-containing lipids), the total polar lipid fraction was subjected to sequential degradation by the procedures of Sheltawy (24). Four fractions were quantified in this study by phosphorus analysis on aliquots of: *1*) water-soluble products resulting from the initial treatment with mild alkali (acyl ester bonds are broken), *2*) the remaining lipid material (contains those with alkyl-ether linkages, alkenyl-ether linkages and sphingolipid amide bonds), *3*) water-soluble products released after the subsequent mild acid treatment of the lipids remaining after mild alkali treatment (alkenyl-ethers are hydrolyzed), and *4*) the lipids remaining after mild acid hydrolysis (alkyl ethers and sphingolipid).

Preparation of diglycerides

Diglycerides of the total polar lipid fraction and of purified individual phospholipids were prepared by phospholipase C digestion procedures previously detailed (25). The lipids were dissolved in $CHCl_3$–MeOH 2:1 (v/v), then 5–20 mg lipids was transferred to 50-ml glass centrifuge tubes and dried under N_2. After the addition of sodium deoxycholate and 3-(N-morpholino)propane sulfonic acid (MOPS) buffer, the mixture was evenly dispersed by sonication for 15 min in an ultrasonicator bath (Mettler Electronics Corp., Anaheim, CA). The enzyme, *Bacillus cereus* phospholipase C (Sigma Chemical Co., St. Louis, MO), was pre-incubated with $CaCl_2$, then added to the dispersed lipid suspension. Hydrolysis was allowed to proceed for 1.5 h at 37°C with vortex agitation of the suspension every 30 min. After digestion, $CHCl_3$ and MeOH were added to the aqueous reaction mixture to form a biphasic partition (22). The lower organic phase containing the diglycerides was recovered and dried.

Preparation of glyceryl ethers

Diglycerides were transferred to 20-ml screw-capped (Teflon-lined) glass tubes and were saponified with 0.6 N KOH in 90% MeOH at 65°C for 1 h. After cooling to room temperature, the glyceryl ethers were recovered by three petroleum ether extractions (20, 26). After drying the glyceryl ether extract, the sample was treated with 0.3 ml of Sil-Prep (Alltech Associates, Inc., Deer-

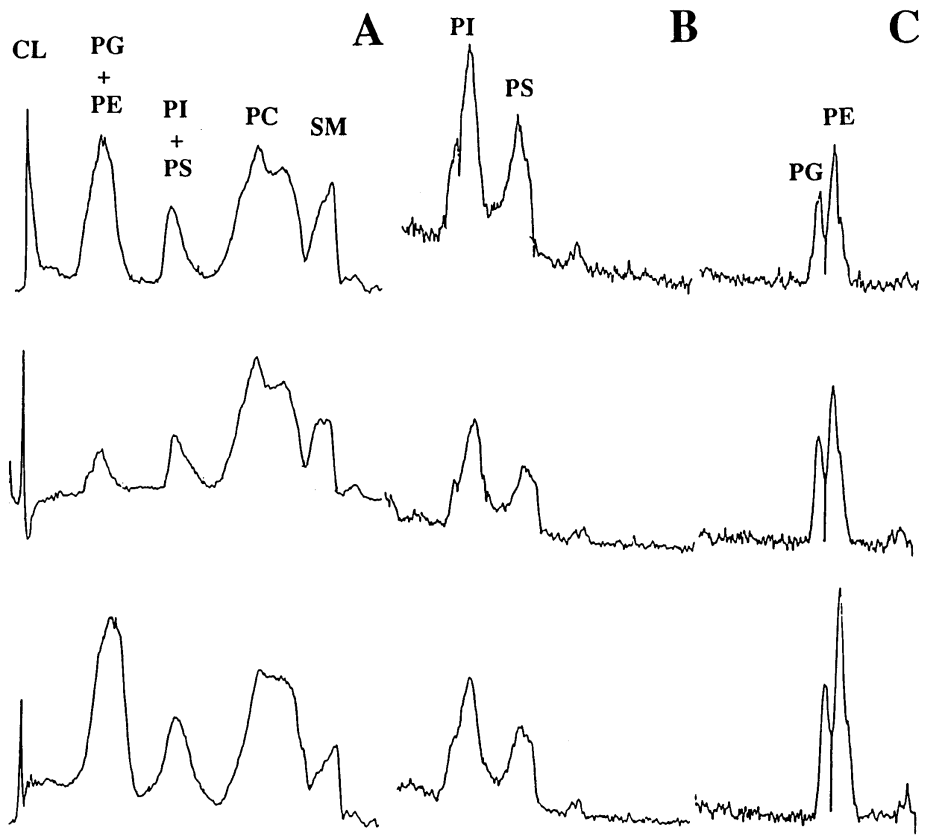

Fig. 1. Densitometric tracings of TLC isolations of the major phospholipid classes in the polar lipid fraction. CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; O, origin. Top, *P. carinii*; middle, lungs from immunosuppressed, uninoculated rats; bottom, lungs from normal, untreated rats. A: Preparative TLC using glass-backed Silica G plates and SS #1 did not sufficiently separate PI from PS and PE from PG. B: After preparative TLC on glass-backed TLC plates and SS #1, material in the band containing PI + PS was separated on Al-backed analytical plates using SS #1. C. After preparative TLC on glass-backed TLC plates and SS #1, the material in the bands containing PE + PG was separated on Al-backed plates using SS #2.

field, IL) at 65°C for 30 min to convert them to their trimethylsilyl (TMS) derivatives. The sample was dried and redissolved in hexane for further analyses.

Isolation of individual TMS glyceryl ethers and determination of double bond positions

Each TMS glyceryl ether species was isolated by preparative GLC on a Hewlett-Packard 5830A GLC using a 6 ft × 0.25-inch i.d. nickel column packed with 10% EGSS-X (Applied Sciences Lab., State College, PA) with a split ratio of 94:6. The separations were accomplished isothermally at 200°C with a He gas flow rate of 24 ml/min. The TMS glyceryl ethers were collected by condensation inside glass wool-plugged Pasteur pipettes at the collection port. The sample was washed from the pipette with $CHCl_3$–MeOH 2:1 (v/v) and then dried.

Oxidation of the double bonds of the monounsaturated TMS glyceryl ethers was done by the method of Scheuerbrandt and Bloch (27) using sodium periodate, potassium permanganate, and potassium carbonate in *t*-butanol. The oxidation reaction was allowed to proceed for 2 h with vortex mixing, then sodium bisulfite was added. After acidification, the monocarboxylic acids resulting from double bond cleavage were extracted into petroleum ether. The acids were converted to their methyl ester derivatives, as described above, then analyzed by GLC using authentic C_7 and C_9 monocarboxylic acid methyl ester standards.

Quantitation of glyceryl ethers

A Hewlett-Packard 5890 GC equipped with a 30 m, 0.32 mm i.d. fused silica capillary column coated with 0.25 μm of DB-1 (J & W Scientific, Folsom, CA) was used for GLC quantitation of derivatized glyceryl ethers and fatty acids. For TMS glyceryl ether analyses, the oven temperature was programmed to increase at a rate of 45°C/min from 40°C to 220°C. The final temperature was maintained for 30 min. Injector temperature was 250°C; flame ionization detector (FID) temperature was 275°C; He carrier gas flow rate was 1 ml/min. Analyses were conducted in the split mode at a 50:1 ratio with injections of 1–2 μg of the samples. Retention times were compared to those of authentic batyl, chimyl and selachyl alcohols (Sigma). Relative retention times (RRT) of peaks were calculated by comparison to the retention time of batyl alcohol.

For GLC analyses of the plasmalogen fraction obtained by sequential degradation of total *P. carinii* polar lipids (see above), the putative plasmalogens in the sample were converted to their isopropylidene derivatives (20, 26). This sample was analyzed isothermally at 180°C on a 30′ × 0.015 mm i.d. SP 2330 column.

To quantify glyceryl ether concentrations, an aliquot of a phospholipid sample was subjected to an ashing method for the release of inorganic phosphate (P_i) which was then quantified spectrophotometrically (28). Peak areas of glyceryl ethers were

compared to that of a known amount of an internal standard (2-O-hexadecyl glycerol) which was added to samples prior to phospholipase C digestion.

Free fatty alcohols

The long chain alcohol fraction was isolated from the neutral lipid fraction by TLC using the solvent system petroleum ether–diethyl ether–acetic acid 80:20:1 (v/v/v; SS #3). Lipid bands were visualized with I_2 vapor. After eluting material from the band scraped off the TLC plate, the fatty alcohols were converted to their trimethylsilyl derivatives with *bis* (trimethylsilyl)trifluoroacetamide (BSTFA, Sigma Chemical Co., St. Louis, MO). They were quantified by GLC using a 30 m × 0.32 mm i.d. capillary column coated with a 0.25 μm DB-1 (J & W Scientific, Folsom, CA) in a Hewlett-Packard 5890 Series II GLC (Hewlett-Packard, Palo Alto, CA). Initial temperature was held for 2 min at 180°C, then increased to 220°C at 2°/min. The injection and FID temperatures were both set at 250°C. Helium was used as the carrier gas; flow rate was 1 ml/min. Chromatography was done in the split mode at a 50:1 ratio or in the splitless mode.

Mass spectrometry

The structures of TMS glyceryl ethers prepared from *P. carinii*, normal lungs, and lungs from immunosuppressed uninfected rats were identified by high resolution MS. A Kratos MS-80 MS (Manchester, U.K.) was interfaced with a Carla Erba series 4160 GLC equipped with a 30 m (0.53 mm i.d.) DB-1 capillary column (1.5 μm film thickness). Samples were introduced by cold on-column injection at an initial oven temperature of 40°C. The oven temperature was increased to 220°C, then, after 4 min from the time of sample injection, the temperature was increased to 280°C at a rate of 3°C/min. Electron impact (EI) spectra were continuously collected and processed by a DS-55 data system based on a Data General NOVA-4X. Structural identities of the glyceryl ethers were determined by comparison with the mass spectral bank of the data system based on their agreement of fragmentation patterns, elemental composition, and molecular masses.

Liquid secondary ion MS (LSIMS; fast atom bombardment, FAB-MS) analysis was done on underivatized authentic PAF (Avanti Polar Lipids, Inc., Alabaster, AL) using glycerol as a matrix. Spectra were also obtained for material in a TLC band present in the lipids of *P. carinii* and lungs from immunosuppressed uninfected rats with the same R_f value as PAF. The sample probe was introduced through the vacuum lock. The cesium gun and power supply were from Phrasor (Phrasor Scientific Inc., Duarte, CA).

Definitive structural identification of trimethylsilyl (TMS) derivatives of fatty alcohols was done by high resolution electron-impact GLC-MS on a Kratos MS-80 instrument (Kratos, Manchester, U.K.) interfaced with a Carlo Erba 4160 GLC equipped with a DB-1 capillary column.

Nuclear magnetic resonance (NMR) spectrometry

The possible presence of plasmalogens was evaluated by ^{1}H-NMR. Total *P. carinii* phospholipids were first subjected to mild alkaline hydrolysis to remove ester-linked fatty acids and water-soluble products. Beef brain PE plasmalogen (90% purity, Serdary Research Lab., Inc., London, Ontario, Canada) was used as an alkenyl ether authentic standard. One mg of *P. carinii* and the authentic standard sample were dissolved in 0.5 ml of deuterated chloroform. Analysis (128 scans) was performed on a Bruker AMX400-MHz NMR spectrometer (Bruker Instruments, Billerica, MA). If plasmalogens were present, a vinyl ether peak would be expected at 5.8 (*cis*) or 6.0 (*trans*) ppm.

RESULTS

Identification of glyceryl ethers

Four glyceryl ethers were detected in the phospholipids of *P. carinii* by GLC of TMS derivatives (**Fig. 2**). The same species were also present in the lipids of normal lungs and lungs of immunosuppressed uninfected rats. They were identified as 1-O-octadecyl glycerol (batyl alcohol), 1-O-octadec-9-enyl glycerol (selachyl alcohol), 1-O-hexadecyl glycerol (chimyl alcohol), and 1-O-hexadec-9-enyl-glycerol. The TMS glyceryl ethers were characterized by the following GLC relative retentions times ($RRT_{batyl\ alcohol}$): batyl alcohol, 1.00; selachyl alcohol, 0.93; chimyl alcohol, 0.62; and 1-O-hexadec-9-enyl glycerol, 0.58.

Structural identities of these lipids were verified by GLC-MS. The TMS derivatives of chimyl, batyl and selachyl alcohols from *P. carinii* had mass spectra identical to those of authentic standards (**Fig. 3**) and were in agreement with the data system bank of mass spectra. An authentic standard of 1-O-hexadec-9-enyl glycerol was not available and its spectrum was not in the data system bank. Hence its identity was based on its fragmentation pattern by EI-MS (**Fig. 4**), and the molecular mass and elemental composition obtained by high resolution MS (**Table 1**). The samples were also converted to diacetyl derivatives. The mass spectra obtained, both by EI-MS and chemical ionization-MS matched those of the standards (not shown).

The double bond positions in the two unsaturated alcohols were further demonstrated to occur between C-9 and

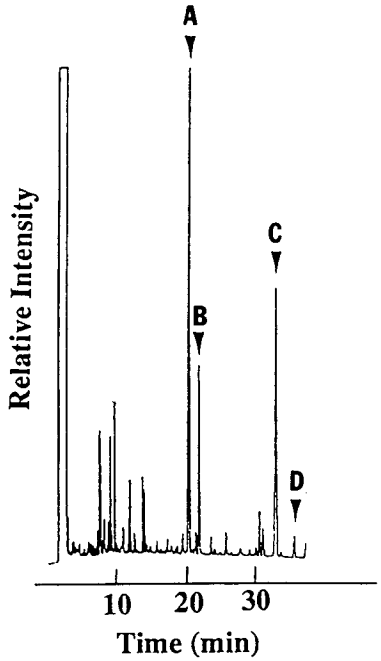

Fig. 2. Separation by GLC of glyceryl ether TMS derivatives prepared from the *P. carinii* total phospholipid fraction. 1-O-hexadecyl glycerol (B), 1-O-octadec-9-enyl glycerol (C), and 1-O-octadecyl glycerol (D) were initially identified by their retention times compared with those of authentic standards. These three components plus 1-O-hexadecyl-9-enyl glycerol (A) were further identified by GLC-high resolution electron-impact MS.

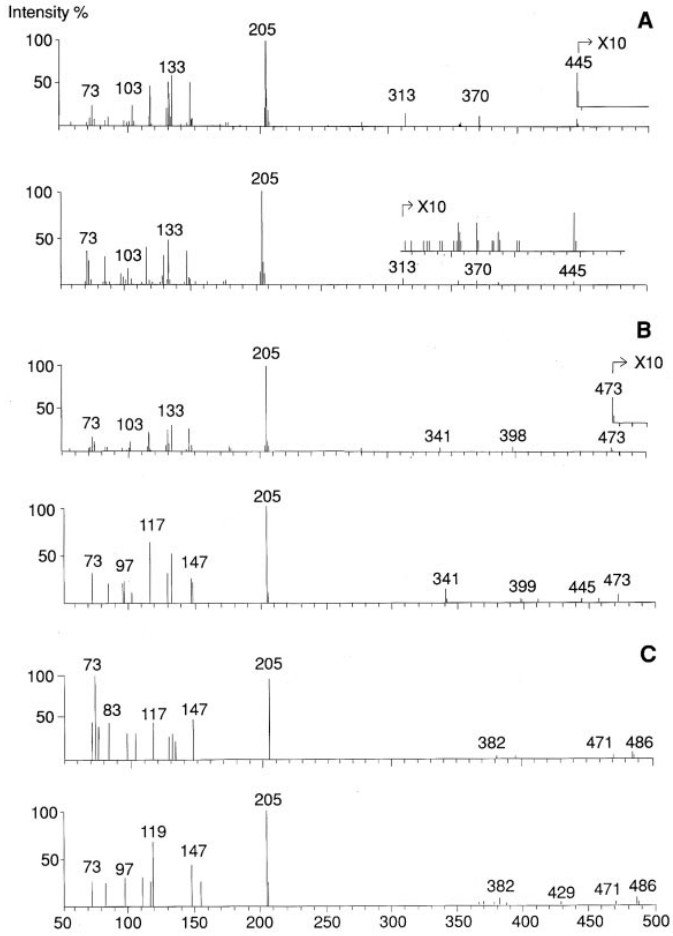

Fig. 3. Mass spectrometry of TMS derivatives of glyceryl ethers. The spectra of these TMS derivatives were identical to the TMS derivatives of authentic standards. A: 1-O-hexadecyl glycerol from *P. carinii* (bottom) and authentic standard (top). B: 1-O-octadecyl-glycerol from *P. carinii* (bottom) and authentic standard (top). C: 1-O-octadecyl-9-enyl glycerol from *P. carinii* (bottom) and authentic standard (top).

C-10 of both long chain alcohols. After oxidation, analysis by GLC of the TMS derivatives showed two major components. One had the same retention time as the fragment obtained from cleavage of the double bond of authentic selachyl alcohol (**Fig. 5**). A seven-carbon monocarboxylic acid fragment resulted from the cleavage of the double bond of 1-O-hexadec-9-enyl glycerol, indicating that the double bond in this long chain alcohol was also at C-9. Peak enhancement of the early eluting component was observed upon cochromatography with authentic heptanoate methyl ester.

TABLE 1. Elemental compositions of the major ion and ion fragments of 1-hexadec-9-enyl glycerol-2,3-trimethylsilyl ether

Experimental Mass	Theoretical Mass	Elemental Composition
458.3613	458.3611	$C_{25}H_{54}O_3Si_2$
311.2402[a]	311.2406	$C_{18}H_{35}O_2Si$
205.1115	205.1080	$C_8H_{21}O_2Si_2$
103.0596	103.0579	$C_4H_{11}OSi$
73.0474	73.0505	C_3H_9Si

[a] $[M^{+\cdot} - OSi(CH_3)_3 - C_4H_9]^+$.

Alkenyl ethers (plasmalogens) and platelet activating factor (PAF)

The sequential degradation of the *P. carinii* total phospholipid fraction indicated that 97% of the phosphorus was released in the water-soluble fraction after mild alkaline hydrolysis. After mild acid hydrolysis of the alkali-treated lipids, there was less than 1% of the remaining phosphorus in the water-soluble fraction which is expected to contain plasmalogen P (alkenyl ethers). However, in the GLC analysis of the plasmalogen fraction, no ether peaks were detected. The absence of plasmalogens was further confirmed by ^{1}H-NMR analysis of total *P. carinii* phospholipids. The vinyl ether peaks of the authentic standard were detected at 5.8919 (*cis*) and 5.9074 (*trans*) ppm, but these peaks were absent in the spectrum of the *P. carinii* sample (**Fig. 6**).

A minor TLC band with R_f of 0.20 was present in the lipids of *P. carinii* and lung controls which comprised 1–3% of the total phospholipids. This band had the same R_f value as authentic PAF and stained with the Dragendorff reagent. The material eluted from this band was subjected to LSIMS and compared with that of authentic PAF. The

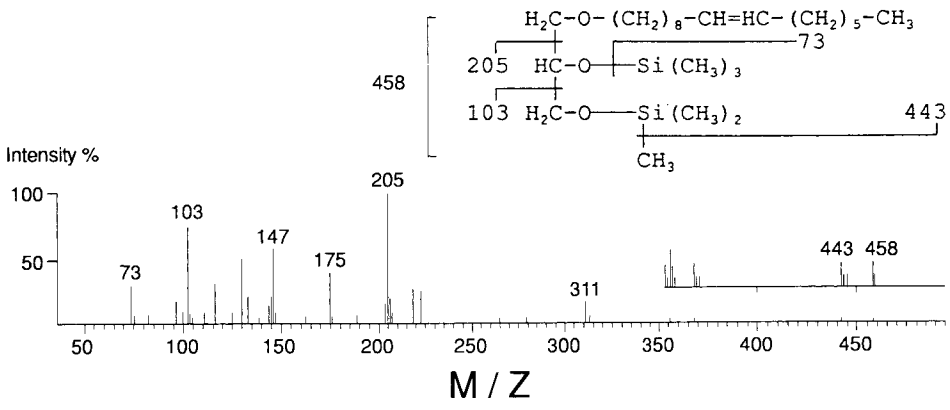

Fig. 4. High resolution mass spectrum and fragmentation pattern of TMS derivative of 1-O-hexadec-9-enyl glycerol from *P. carinii*.

LSIMS spectral data indicated that PAF was not detectable in the material eluted from that 1-D TLC band.

Quantitation of glyceryl ethers

Analysis by sequential degradation of the total *P. carinii* polar lipid fraction indicated that a total of about 5% of the polar lipid fraction consisted of alkyl ethers, alkenyl ethers, and sphingolipids (alkaline-stable bonds). Thin-layer chromatographic and phosphorus analyses of isolated phospholipids demonstrated that ether lipids were not in high concentrations as they can occur in some other tissues. Quantitation of glyceryl ethers by the procedure in which phospholipids were first converted by phospholipase C to diglycerides demonstrated that approximately 3% of the total polar lipid fractions from *P. carinii* and lung samples contained ether bonds (**Table 2**).

Corticosteroid treatment itself altered the glyceryl ether content of rat lungs. A highly significant increase ($P < 0.005$) in the concentration of alkylacyl PI resulted from methylprednisolone immunosuppression. The ether concentrations also increased in lyso PC and PE. Compared with lungs from immunosuppressed uninfected rats, *P. carinii* organisms had a higher concentration of alkylacyl PC and lower concentrations of alkyacyl PE, alkyacyl PI, and lyso alkyl PC.

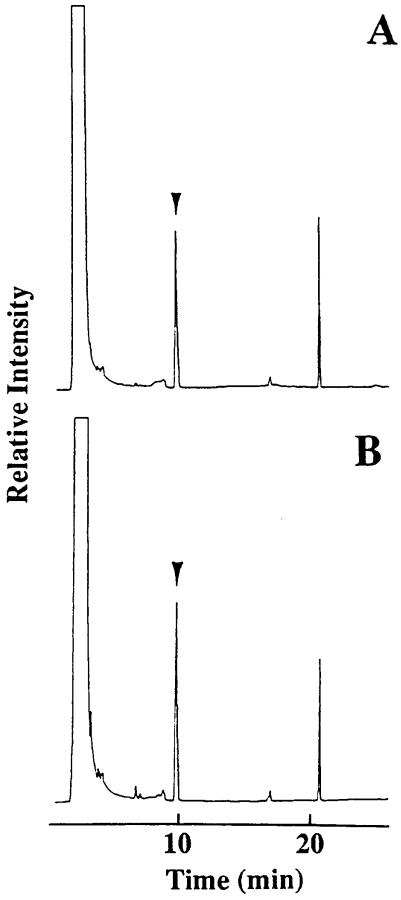

Fig. 5. End fragment analysis of monounsaturated *P. carinii* glyceryl ethers by GLC after oxidation and derivatization. A: Two major components were produced upon oxidation of the total glyceryl ether fraction. These had the same retention times (9.950 min and 21.299 min) as those of authentic derivatized n-heptanoic acid and n-nonanoic acid. B: Cochromatography of *P. carinii* total glyceryl ether end fragment derivatives with authentic heptanoic acid methyl ester demonstrated enhancement of the earlier eluting peak. The retention time of the enhanced peak was 9.939 min; the second major peak had a retention time of 21.283 min.

Analyses of the glyceryl ether compositions of the total polar lipid fraction and the major phospholipid classes demonstrated that 1-O-hexadecyl glycerol (chimyl alcohol) was the dominant ether lipid present in *P. carinii* and the lungs of normal rats and corticosteroid-treated rats (**Tables 3–5**). However, large variations in different samples were observed. In a few samples, unsaturated alcohols were present in higher concentrations than the saturated acids of the same chain length, and in some cases, minor components were not detected or integrated by GLC analyses due to small sample sizes. In the present study, only the major phospholipid classes in the polar lipid fraction were analyzed. Other (e.g., cardiolipin) minor components are present in the specimens (6). Hence the values of the total polar lipid fraction would include glyceryl ethers that may exist in the minor components that were not individually analyzed.

Individual phospholipid classes contained all four species of fatty alcohols, and they differed in the relative concentrations of each. Unlike lung PC, lung lyso PC had high concentrations of 1-O-hexadec-9-enyl glycerol, which was observed in both normal rats and corticosteroid-treated rats. In contrast, the glyceryl ether compositions of both *P. carinii* PC and lyso PC were similar.

Free fatty alcohols

All long chain alcohols detected in lungs of normal rats, immunosuppressed uninfected rats, and *P. carinii* were saturated species (**Table 6**). Hexadecanol (16:0) was the dominant free fatty alcohol in all samples; molecules with chain lengths up to 26 carbons were present in the free fatty alcohol fraction.

DISCUSSION

Glyceryl ethers of the major phospholipid classes

Daigne et al. (29) previously reported that Wistar rat lung PC and PE contained ether lipids. Our results obtained on Lewis rat lungs were similar to their values for percent alkyl acyl species in those two lipid classes. Analyses of additional phospholipid classes performed in the present study showed that PS and especially PI contained much higher proportions of glyceryl ethers than did PC and PE.

Methylprednisolone was previously shown to alter the relative amounts of phospholipid classes in rat lungs with a decrease in percentages of PE and increases in PI and lyso PC (6). Because PI concentrations of whole lung phospholipids increased from 2% to 4% in response to corticosteroid treatment (6), the increased percentage of alkyl acyl species in PI (from 12% to 45%) found in this study indicates that the increase in alkyl acyl PI was dra-

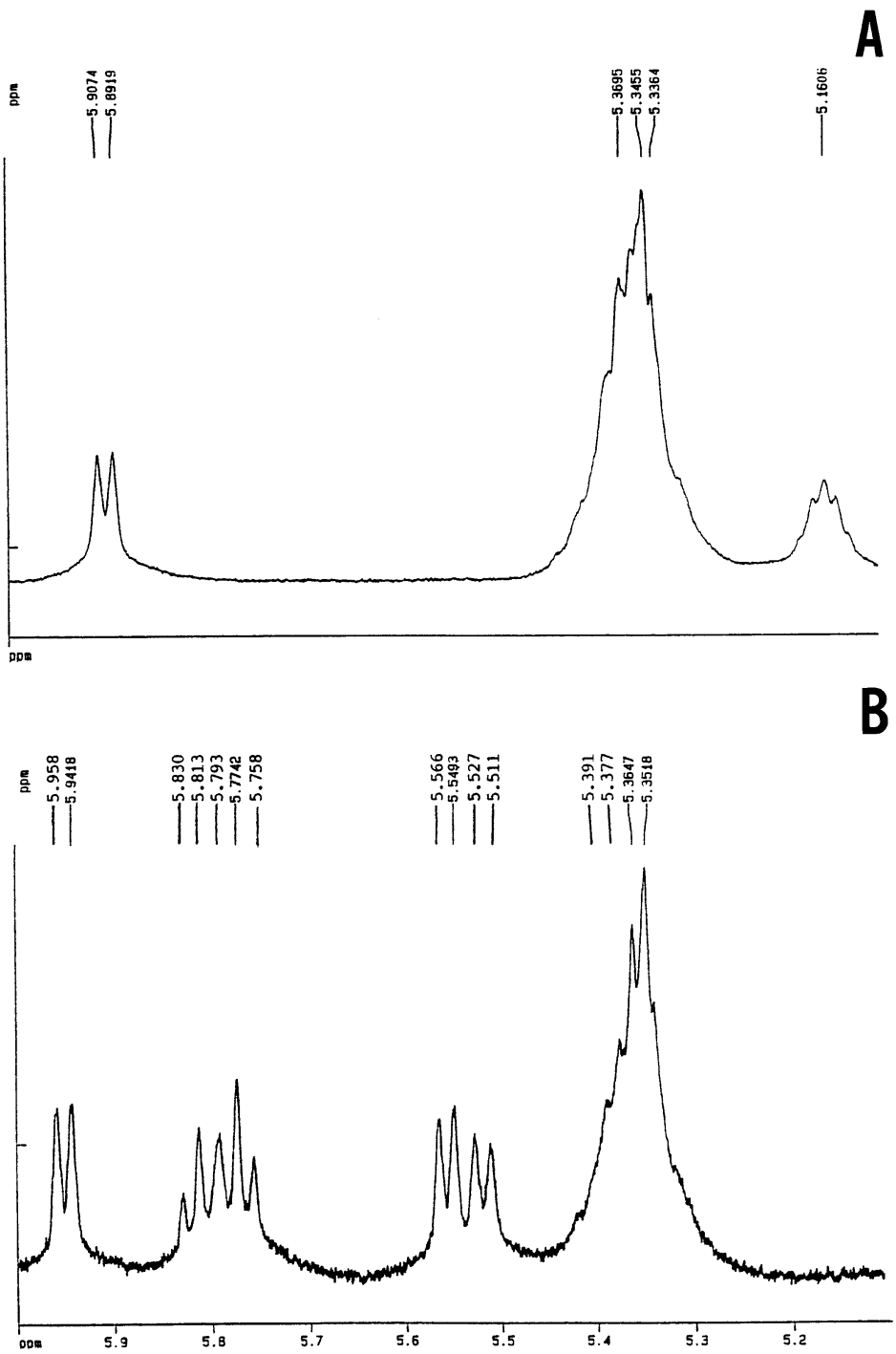

Fig. 6. Nuclear magnetic resonance spectrum of alkali-stable products. A: Alkali-stable products derived from authentic standard of beef brain phosphatidylethanolamine plasmalogens. B. Alkali-stable products derived from the total polar lipid fraction of *P. carinii* organisms.

matic. It has been shown that corticosteroids increase lung surfactant secretion by alveolar Type II cells (30). It is not known whether the changes in the glyceryl ethers of whole lung lipids observed are due to increased surfactant secretion or to other changes in lung cells.

Methylprednisolone also caused a significant increase in lyso PC concentrations of whole lung lipids (6) ($P < 0.005$). Thus, the increased ether content in lyso PC observed in this study indicates that there was a considerable increase in lyso alkyl PC in the lung in response to steroid treatment. It is interesting to note that the glyceryl ether compositions of lung PC and lyso PC were quite different. Lyso PC in lungs from both normal and methylprednisolone-treated rats contained much higher 1-O-hexadec-9-enyl glycerol than PC. In lungs from normal untreated rats, lyso PC contained much less 1-O-

TABLE 2. Glyceryl ether contents of the total polar lipid fraction and the major phospholipid classes of *Pneumocystis carinii carinii* and whole lungs from normal and immunosuppressed uninfected rats

	Normal			Immunosuppressed Uninfected			*Pneumocystis carinii*	
Phospholipid	μmol/μmol	n	P^a	μmol/μmol	n	P^b	μmol/μmol	n
TPL	0.037 ± 0.006	5	<0.02	0.056 ± 0.005	5	<0.20	0.048 ± 0.007	6
PC	0.016 ± 0.003	5	<0.30	0.021 + 0.007	5	<0.01	0.054 ± 0.011	6
PE	0.060 ± 0.17	5	<0.01	0.130 ± 0.050	5	<0.05	0.050 ± 0.009	6
PI	0.121 ± 0.050	5	<0.005	0.454 ± 0.089	5	<0.05	0.306 ± 0.036	6
PS	0.120 ± 0.026	5	<0.30	0.101 ± 0.020	5	<0.20	0.075 ± 0.023	6
PG	0.029 ± 0.006	5	<0.10	0.048 ± 0.012	5	<0.30	0.056 ± 0.010	6
LysoPC	0.015 ± 0.004	4	<0.05	0.025 ± 0.003	6	<0.05	0.014 ± 0.004	6

Values are given as means ± SEM, TPL, total lipid fraction eluted from Unisil columns with MeOH; PC, phosphatidylcholine; PE, phosphatidyethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; Lyso PC, lysophosphatidylcholine.

[a] Normal vs immunosuppressed uninfected.

[b] Lungs from immunosuppressed uninfected rats versus *P. carinii*.

hexadecyl glycerol than PC. These data suggest that normally, much of the lyso alkyl PC molecules in the lung are not directly derived from PC by phospholipase A_2 action. As lyso PC is normally found in relatively high concentrations in lung compared to most other tissues, the de novo biosynthesis of this lipid, which has potent surfactant properties, may be well regulated in the lung. Lyso PC probably plays a role in reducing surface tension of the alveolar lining fluid.

Plasmalogens

The lungs of mongrel dogs were reported to contain high levels of plasmalogens, especially in phosphatidylethanolamine which was reported to be 67% alkenyl lipids (31). Also, Diagne et al. (29) reported that Wistar rat lung PE contained almost as much alkenyl as diacyl, and only trace amounts of alkyl acyl molecules. In that study, two-dimensional HCl reaction TLC methods were used to separate these compounds. Our results suggest that mammalian species and rat strain differences may exist as we were not able to detect alkenyl phospholipids in the lipids of lungs from the female Lewis rats. In the present study, several independent analytical methods were used. Both sequential degradation and NMR techniques used in this study indicated that plasmalogens were not present in detectable amounts. In previous studies conducted in our labs, we found that two-dimensional reaction TLC (32) and sequential degradation (24) gave different results. It cannot be ruled out that the methods used for plasmalogen analysis, rather than species and strain differences, are the major reasons for these apparent discrepancies.

Platelet activating factor

Prevost, Cariven, and Chap (33) did not detect PAF in alveolar lavage fluid of normal Wistar rat, but relatively high levels of PAF and especially lyso alkyl PC appeared after exposing the animals to hypoxic conditions. These workers concluded that the increase in lyso alkyl PC resulted from the removal of the acetyl group of PAF. However, they did not examine whether the increase occurred by the removal of the fatty acid from 1-alkyl-2-long chain acyl PC (which would produce similar lyso compounds) or that de novo synthesis of lyso alkyl PC occurred. Because acute PcP is characterized by hypoxia, we analyzed our samples for PAF and changes in lyso alkyl PC. We showed that PAF was absent in the phospholipids of whole lungs from normal and methylprednisolone-treated rats, as well as in *P. carinii*. It was previously shown that the concentration of lyso PC in Lewis rat lungs increased in response to methylprednisolone treatment, and that *P. carinii* had more lyso PC than the lungs from normal rats or immunosuppressed uninfected rats (6). In the present study, we found that lyso PC of *P. carinii* organisms and the lung controls had the same percentage of glyceryl ethers.

TABLE 3. Glyceryl ether compositions of the total polar lipid fraction and the major phospholipid classes of lungs from normal rats

Glyceryl Ether	TPL	PC	PE	PI	PS	PG	Lyso PC
				weight %			
1-O-hexadecyl glycerol	67.4 ± 4.3	84.1 ± 1.6	70.8 ± 5.0	78.1 ± 2.2	74.8 ± 8.1	73.1 ± 3.2	49.2 ± 1.6
1-O-hexadec-9-enyl glycerol	12.2 ± 3.4	4.5 ± 1.8	4.7 ± 2.5	10.4 ± 1.1	11.4 ± 0.7	8 .0 ± 1.2	35.3 ± 5.5
1-O-octadecyl glycerol	14.1 ± 2.7	8.4 ± 2.4	22.2 ± 1.6	11.1 ± 1.2	13.7 ± 3.5	14.7 ± 3.6	11.4 ± 1.6
1-O-octadec-9-enyl glycerol	4.6 ± 1.5	3.3 ± 0.7	3.9 ± 1.6	0.4 ± 1.2	trace	4.3 ± 2.2	4.2 ± 3.3
% Saturated	81.5	92.5	93.0	89.2	88.5	87.8	60.6

Values given as means ± SEM; n = 4–6 separate biological specimens. TPL, total polar lipid fracton eluted with MeOH from Unisil columns; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol.

TABLE 4. Glyceryl ether compositions of the total polar lipid fraction and the major phospholipid classes of lungs from immunosuppressed uninfected rats

Glyceryl Ether	TPL	PC	PE	PI	PS	PG	Lyso PC
				weight %			
1-O-hexadecyl glycerol	70.2 ± 7.9	75.7 ± 5.1	68.8 ± 7.8	83.0 ± 3.5	73.3 ± 3.5	68.9 ± 4.7	71.6 ± 7.5
1-O-hexadec-9-enyl glycerol	12.1 ± 3.2	6.5 ± 1.5	6.8 ± 2.1	7.6 ± 3.3	9.7 ± 2.5	3.5 ± 0.3	23.1 ± 5.6
1-O-octadecyl glycerol	11.3 ± 2.2	10.1 ± 0.6	21.9 ± 1.5	12.2 ± 3.1	17.1 ± 3.8	20.1 ± 2.8	6.4 ± 2.0
1-O-octadec-9-enyl glycerol	4.5 ± 1.1	7.8 ± 1.6	1.7 ± 1.2	0.4 ± 0.8	0.5 ± 0.2	7.1 ± 0.1	0.6 ± 0.6
% Saturated	81.5	85.8	90.7	95.2	90.4	89.0	78.0

Values given as means ± SEM; n = 6 separate biological specimens. TPL, total polar lipid fraction eluted with MeOH from Unisil columns; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; Lyso PC, lysophosphatidylcholine.

The glyceryl ether composition of *P. carinii* PC and lyso PC were similar (Table 5), suggesting that 1-alkyl lyso PC was derived from 1-alkyl-2-acyl PC by phospholipase A_2 action, or 1-alkyl lyso PC was acylated to form PC. Interestingly, in contrast to *P. carinii*, the glyceryl ether compositions of PC and lyso PC of the lungs from both normal rats and immunosuppressed uninfected rats differed (Tables 3 and 4). This suggests that *P. carinii* either selectively scavenges these lipids from the host, tailors its ether lipids for optimal functioning of its membranes, or de novo synthesizes ether lipids. The differences in glyceryl ether composition of PC and lyso PC in the lungs of normal rats and methylprednisolone acetate-treated rats suggests that in the lung, much of PC and lyso PC molecules are not formed by precursor-product reactions. As lyso lipids have potent surfactant properties, we suggest that specific alkyl lyso PC may be de novo synthesized and secreted into the alveolar lining fluid and plays a role in reducing surface tension.

Free fatty alcohols

The fatty alcohols are minor lipids comprising less than 1% of the neutral lipid fraction of *P. carinii carinii* (3). The free fatty alcohol composition of *P. carinii* was qualitatively and quantitatively quite similar to those in the rat lung controls; all were saturated. Long chain alcohols are precursors of wax esters and glyceryl ethers; wax esters were not detected in *P. carinii* or rat lung controls (E. S. Kaneshiro, J. E. Ellis, and K. A. Kallam, unpublished results).

Of the four glyceryl ethers, 1-O-hexadecyl glycerol was the dominant species in *P. carinii* and rat lungs, which is consistent with hexadecanol comprising the highest percentage of their free fatty alcohol fractions. The absence of monounsaturated precursor free long chain alcohols suggests that the alcohol moiety of glyceryl ethers undergoes modification by desaturase activity. The presence of a wide range of fatty alcohols (with 10 to 26 carbons) in the free fatty alcohol fraction suggests that fatty alcohols serve other metabolic or physiological functions other than precursors of glyceryl ethers and wax esters.

Lipid biochemistry of the opportunistic pathogen *Pneumocystis carinii*

The development of a purification protocol has enabled the elucidation of the biochemical nature of *P. carinii*, especially with respect to its lipids. Total cellular and total extracted lipid fatty acid compositions have been reported (9, 34), and studies on uptake and metabolism have been initiated (35, 36). The major phospholipid (6, 37) and neutral lipid classes (3) and their fatty acid compositions have been examined in detail (7, 9). The absence of detectable phosphonolipids (6, 38) has been documented (6). The major ubiquinone homologs in the organism have been identified (3), and de novo biosynthetic pathways are being examined. Several unique sterols have been detected (4, 8, 38, 39), which are being studied in greater detail. This report represents the first study of the glyceryl ethers and free fatty alcohols in this important AIDS-associated opportunistic pathogen.

TABLE 5. Glyceryl ether compositions of the total polar lipid fraction and the major phospholipid classes of *Pneumocystis carinii carinii* organisms

Glyceryl Ether	TPL	PC	PE	PI	PS	PG	Lyso PC
				weight %			
1-O-hexadecyl glycerol	70.8 ± 5.5	78.5 ± 2.2	79.1 ± 2.8	66.7 ± 2.7	63.4 ± 2.4	66.7 ± 5.5	75.4 ± 3.5
1-O-hexadec-9-enyl glycerol	15.1 ± 3.3	8.8 ± 1.0	9.4 ± 2.4	15.5 ± 1.7	18.5 ± 5.3	14.9 ± 1.7	6.1 ± 1.0
1-O-octadecyl glycerol	10.9 ± 2.3	9.8 ± 1.9	11.1 ± 2.6	16.1 ± 4.3	16.8 ± 2.6	18.5 ± 2.1	12.4 ± 0.8
1-O-octadec-9-enyl glycerol	3.4 ± 0.9	4.5 ± 1.5	0.4 ± 0.2	1.9 ± 0.4	1.2 ± 0.7	2.2 ± 1.4	6.2 ± 3.4
% Saturated	81.7	88.3	90.2	82.8	80.2	85.2	87.8

Values given as means ± SEM; n = 6–7 separate biological samples. TPL, total polar lipid fraction eluted with MeOH from Unisil columns; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; Lyso PC, lysophosphatidylcholine.

TABLE 6. Composition of the long chain alcohol fraction of lipids extracted from the lungs of normal and immunosuppressed uninfected rats and from *P. carinii carinii* organisms

Fatty Alcohol	Rat Lungs		
	Normal	Immunosuppressed Uninfected	*Pneumocystis carinii*
		weight %	
10:0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
11:0	0.2 ± 0.2	0.8 ± 0.4	0.6 ± 0.3
12:0	0.4 ± 0.2	2.3 ± 0.8	1.1 ± 0.3
13:0	1.4 ± 0.4	0.3 ± 0.1	1.8 ± 0.5
14:0	2.9 ± 1.0	3.7 ± 0.7	1.6 ± 0.4
15:0	5.0 ± 1.9	2.3 ± 0.5	1.4 ± 0.5
16:0	49.6 ± 2.8	49.6 ± 2.4	50.5 ± 2.2
17:0	4.9 ± 1.0	4.4 ± 1.9	2.8 ± 0.5
18:0	8.0 ± 1.4	9.6 ± 2.1	7.6 ± 1.2
19:0	2.0 ± 0.3	3.6 ± 0.6	2.4 ± 0.4
20:0	5.3 ± 0.7	4.1 ± 0.7	6.1 ± 0.8
21:0	1.6 ± 0.6	0.8 ± 0.2	2.4 ± 0.5
22:0	14.7 ± 0.9	14.6 ± 0.9	16.7 ± 0.9
23:0	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
24:0	2.8 ± 0.8	2.9 ± 0.8	3.3 ± 0.6
26:0	1.1 ± 0.6	0.8 ± 0.4	1.4 ± 0.8
n	8	9	13

Values given as means ± SEM. *P* values between lungs of normal versus immunosuppressed rats: 12:0, <0.04; lungs of normal rats versus *P. carinii*: 15:0, <0.04; lungs of immunosuppressed rats versus *P. carinii*: 13:0, <0.05, 21:0, <0.05.

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