Fatty Acid Composition of the Major Phospholipids of *Pneumocystis carinii carinii*: Comparison with Those in the Lungs of Normal and Methylprednisolone-Immunosuppressed Rats

ZENGKUI GUO,¹ DAVID H. BEACH,² AND EDNA S. KANESHIRO^{1*}

Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221,¹ and

Department of Microbiology and Immunology, State University of New York Health Science Center, Syracuse, New York 13210²

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Large numbers of viable organisms can be isolated from the corticosteroid-immunosuppressed rat model of *Pneumocystis carinii* pneumonia. With the development of purification protocols that provide organism preparations of high purity, meaningful lipid biochemical analyses of this important opportunistic pathogen can now be conducted. The phospholipid class composition of the pathogen was reported earlier, together with observations of changes that occur in the rat lungs in response to methylprednisolone immunosuppression treatment. In this report, analyses of the effects of corticosteroids on the fatty acid compositions of the major lung phospholipids, individually isolated and purified by thin-layer chromatography, were elucidated and quantified by gas-liquid chromatography. In response to methylprednisolone, there was a relative increase in palmitate and there were decreases in several unsaturated fatty acids of the rat whole-lung total polar lipids, leading to a doubling of the saturation index. Reciprocal changes in the relative concentrations of palmitate and stearate in phosphatidylethanolamine, phosphatidylinositol, lysophosphatidylcholine, and cardiolipin were observed, suggesting that there is tight control of acylation of these phospholipids in the lung. Detailed phospholipid fatty acid analyses were also performed with mixed life cycle stages of *P. carinii* organisms. The most abundant phospholipids, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, had much higher concentrations of oleic acid and lower concentrations of palmitate in *P. carinii* than in lung tissue. Sphingomyelin in lung tissue and *P. carinii* differed from the glycerophospholipids by the presence of high levels of saturated C₂₂ and C₂₄ fatty acids. This study represents the most comprehensive fatty acid analysis of rat lung phospholipids and the changes that occur in response to corticosteroid treatment. It is the first report about the fatty acids of individual phospholipids of the opportunistic protist *P. carinii carinii*.

The most prevalent opportunistic infective agent in AIDS patients is *Pneumocystis carinii*, a protist that can also cause disease in other individuals, such as patients undergoing treatment for cancer and organ transplantation, malnourished children, and others with defective or weakened immune systems. Although aggressive prophylaxis with a combination of trimethoprim and sulfamethoxazole has dramatically reduced the number of acute *P. carinii* pneumonia (PCP) cases in the United States and other countries, immunosuppressed individuals remain at risk for infection by this pathogen. *Pneumocystis* infection in humans is probably initiated by the entry of the pathogen into the mammalian lung alveolus. Although this organism has been identified in other organs and tissues (27), it appears to grow at the highest rate in the lungs of immunodeficient mammals. This extracellular parasite attaches to type I alveolar epithelial cells, and as it multiplies, it fills the alveolar lumen. *P. carinii* pneumonia ultimately results in low blood oxygenation and damage to pulmonary tissue (28).

The fluid that lines the lung alveolus (hypophase) contains lung surfactant, which is composed largely of surfactant phospholipids, especially dipalmitoyl phosphatidylcholine (PC) (9, 23). Parasites are known to scavenge lipids and other mole-

cules biosynthesized by their hosts. Species of the genus *Pneumocystis* apparently scavenge large quantities of several lung surfactant lipids (12, 14, 18, 24). However, there is evidence that *P. carinii* is capable of synthesizing certain lipids de novo (4, 7, 12), including some that are not detectable among the lipids of the host lung. Information on the lipid composition and metabolism of *P. carinii* comes mainly from the corticosteroid-immunosuppressed rat model. Because organisms are obtained from these drug-treated animals, it is necessary to examine potential lipid biochemical changes in the lung that might be induced by the corticosteroid treatment itself. The lipids of the lungs of corticosteroid-treated rats can then be appropriately compared with those of the pathogen to determine the lipid profiles and lipid species characteristic of the organism. We previously reported the phospholipid composition of rat-derived *P. carinii*, as well as the biochemical changes induced in whole rat lungs by methylprednisolone (8). A significant reduction in the relative concentration of phosphatidylethanolamine (PE) and increases in phosphatidylinositol (PI) and lysophosphatidylcholine (LPC) resulted as a consequence of corticosteroid treatment. *Pneumocystis* organisms contained relatively lower concentrations of PC and more PI than the lipids of whole lungs from immunosuppressed uninfected rats. Structural identifications of *P. carinii* fatty acids and the fatty acid compositions of total lipids and the major neutral lipid components are presented elsewhere (10a, 11, 14).

* Corresponding author. Mailing address: Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221-0006. Phone: (513) 556-9712. Fax: (513) 556-5280. Electronic mail address: Kaneshir@UCBEH.San.UC.EDU.

TABLE 1. Fatty acid composition of the total polar lipid fraction of *P. carinii* and lungs from normal and immunosuppressed uninfected rats^a

Fatty acid	Wt % of fatty acids from ^b :			
	<i>P. carinii</i>	<i>P</i> ^c	Rat lungs	
			Immunosuppressed uninfected	Normal
14:0	0.2 ± 0.2		1.3 ± 0.4	2.0 ± 0.1
15:0	0.3 ± 0.1		0.7 ± 0.2	0.4 ± 0.1
16:0	25.4 ± 2.5	<0.0001	57.8 ± 2.6	40.0 ± 1.8
16:1	2.2 ± 0.4		2.2 ± 0.5	3.2 ± 0.8
17:0	0.2 ± 0.1		0.4 ± 0.1	0.4 ± 0.1
18:0	12.2 ± 0.6		12.5 ± 3.4	15.8 ± 1.5
18:1	32.0 ± 2.1	<0.0001	7.6 ± 1.0	13.6 ± 2.1
18:2	5.7 ± 0.5	<0.05	3.9 ± 1.0	6.2 ± 0.7
20:0	0.5 ± 0.1		0.3 ± 0.1	0.8 ± 0.1
20:3	5.7 ± 0.1		1.5 ± 0.6	0.7 ± 0.3
20:4	5.2 ± 0.4		6.3 ± 1.0	12.4 ± 0.5
20:5	0.3 ± 0.1		0.8 ± 0.2	1.5 ± 0.2
21:0	1.7 ± 0.2		0.4 ± 0.1	0.2 ± 0.2
22:0	ND ^e		Trace	Trace
22:1	0.9 ± 0.1		0.4 ± 0.2	0.2 ± 0.1
22:4	3.7 ± 0.4	<0.0001	0.9 ± 0.2	0.8 ± 0.1
22:5	ND		1.4 ± 0.2	2.5 ± 0.3
23:0	ND		ND	Trace
24:0	2.3 ± 0.3		Trace	2.0 ± 0.7
24:1	2.4 ± 0.2	<0.0001	0.3 ± 0.1	0.7 ± 0.1
Others ^f	1.9 ± 1.2		2.9 ± 3.3	2.4 ± 1.6
% Sat ^g	42.8		73.4	61.6
S/U ^h	0.737		2.901	1.474

^a Total lipids were extracted and then separated by adsorption column chromatography into two major fractions, the neutral lipid fraction and the polar lipid fraction. The polar lipid fraction contains all of the major phospholipid classes and other minor lipid classes.

^b Except as noted, values are means ± standard errors (*n* = 6).

^c *P* values for comparison of *P. carinii* with immunosuppressed uninfected controls.

^d *P* values for comparison of lungs from immunosuppressed uninfected rats with those from normal rats.

^e ND, not determined.

^f Sum of other minor fatty acids present in the chromatograms.

^g Sum of saturated fatty acids.

^h S/U, saturation index (sum of saturated fatty acids/sum of unsaturated fatty acids).

In the present study, the phospholipids of *P. carinii* were examined in greater detail. Parallel analyses of phospholipids isolated from the lungs of normal and methylprednisolone-treated rats were also performed. The major classes were isolated, and their fatty acid compositions were analyzed to determine whether differences between those of the organism and those of the rat lungs could be detected.

MATERIALS AND METHODS

Organisms and rat lungs. The Boylan and Current (3) induced PCP model was used to obtain *P. carinii carinii* (prototype karyotype) (21) from corticosteroid-immunosuppressed viral antibody-negative Lewis rats (Harlan Sprague-Dawley, Room 202B, Indianapolis, Ind.). All animals were housed in a room fitted with a laminar-flow BioBubble (Bubble Systems/CSA, Inc., Fort Collins, Colo.). Infections were induced by intratracheal inoculation of prefrozen organisms into rats treated with methylprednisolone.

Mixed life cycle stages of *P. carinii* were isolated and purified from the infected lungs by procedures developed for lipid analysis as described previously (15). The purity of the final organism preparation was characterized by several criteria. Light microscopy indicated intact host cells were absent, and values obtained by transmission electron microscopy were conservatively estimated at >95% purity. Enzyme-linked immunosorbent assays of the lung surfactant marker surfactant protein A demonstrated that the organism preparation contained <0.5% contaminating surfactant. Exogenously added stigmasterol was completely removed by the purification procedure, as indicated by undetectable levels of stigmasterol in the final organism preparation. The protein content of pelletable material resulting from processing of *P. carinii*-free rat lungs according to this protocol indicated that the organism preparations could contain no more than 6% non-*P. carinii* protein (15). The organism populations used in the present studies were composed mainly of trophozoite forms with 10 to 30% cysts.

Whole rat lungs were used as controls. Two types of controls were analyzed: lungs from normal rats and lungs from immunosuppressed uninfected rats.

Untreated normal rats and immunosuppressed uninoculated rats were kept under double barrier conditions. They were kept in microisolator cages within the laminar-flow BioBubble. These animals did not develop PCP, as assessed by undetectable organisms in lung impression smears and in process controls described previously (15). The lungs of normal and immunosuppressed uninfected rats were excised, sliced, homogenized, and then disrupted in a French pressure cell as previously described (8).

Extraction of lipids, fractionation, and isolation of individual phospholipids. All organic solvents used for extraction and processing of lipids contained butylated hydroxytoluene to prevent oxidation of the samples. However, the solvent used to dissolve the final sample for gas-liquid chromatographic (GLC) analysis did not contain butylated hydroxytoluene, since the compound is detected as a discrete peak in chromatograms of fatty acid methyl esters (FAME).

Samples of lungs from a single rat and *P. carinii* preparations obtained from a single rat were extracted for total lipids by determining the volume of the aqueous suspension and then adding 3.73 times the volume of a mixture of chloroform (CHCl₃)-methanol (1:2 [vol/vol]) and stirring the suspension for several hours on a magnetic stirrer at room temperature (2). The entire mixture was transferred to a separatory funnel, and appropriate amounts of solvents were added to convert the mixture to a biphasic system to purify lipids from other compounds (6). After phase separation, the lower organic phase was removed, and freshly prepared lower phase solution was added to reextract the upper phase. The lower organic phases were combined, reduced in volume by rotary evaporation (Rotovap; Buchi, Brinkmann Instruments, Westbury, N.Y.), transferred to shell vials with Teflon-lined screw caps, and then dried under a stream of N₂ (N-Evap; Organomation, Berlin, Mass.).

The total lipids extracted from *P. carinii* and rat lungs were then fractionated into neutral and polar lipids by adsorption column chromatography (13). The total lipids were redissolved in CHCl₃ and loaded onto a glass column packed with activated silicic acid (Unisil; Clarkson Chemical Co., Williamsport, Pa.). The neutral lipid fraction was eluted with CHCl₃, and then the polar lipid fraction was stripped off the column with methanol.

The polar lipid fraction was subjected to preparative thin-layer chromatography (TLC) on glass-backed plates coated with 500 μm of silica gel G (Alltech Associates, Inc., Deerfield, Ill.) with CHCl₃-methanol-acetic acid-H₂O (50:25:7:3

TABLE 2. Fatty acid compositions of the major phospholipid classes of whole lungs from normal rats

Fatty acid	Wt % of fatty acids from ^a :							
	LPC	SM	PC	PI	PS	PE	PG	CL
14:0	0.4 ± 0.1	1.4 ± 0.4	1.2 ± 0.5	4.0 ± 1.4	2.0 ± 0.1	2.2 ± 0.4	1.6 ± 1.2	1.5 ± 0.1
15:0	0.5 ± 0.1	1.4 ± 0.7	0.5 ± 0.2	0.9 ± 0.1	0.9 ± 0.4	4.8 ± 0.5	0.9 ± 0.3	1.2 ± 0.2
16:0	29.9 ± 2.4	18.1 ± 4.4	65.6 ± 1.5	23.5 ± 3.2	33.4 ± 1.1	14.9 ± 7.4	51.6 ± 9.6	30.3 ± 8.5
16:1	0.8 ± 0.2	1.0 ± 0.3	2.0 ± 0.4	2.3 ± 0.5	2.4 ± 0.3	0.6 ± 0.1	2.7 ± 0.6	1.5 ± 0.1
17:0	1.4 ± 0.1	0.7 ± 0.4	0.3 ± 0.2	0.8 ± 0.2	1.7 ± 0.6	1.0 ± 0.3	0.7 ± 0.5	1.4 ± 0.2
18:0	33.0 ± 7.1	10.7 ± 1.7	8.0 ± 1.0	48.3 ± 7.4	29.7 ± 3.6	30.7 ± 6.6	11.1 ± 3.1	17.3 ± 2.7
18:1	3.9 ± 1.1	1.7 ± 0.3	8.8 ± 1.4	4.0 ± 1.0	7.3 ± 0.7	12.7 ± 0.8	13.9 ± 2.9	7.9 ± 1.8
18:2	0.8 ± 0.1	0.3 ± 0.1	1.1 ± 0.1	2.4 ± 0.3	1.2 ± 0.1	2.5 ± 0.7	3.5 ± 2.8	1.9 ± 0.2
20:0	2.0 ± 0.4	4.1 ± 0.1	0.5 ± 0.2	1.6 ± 0.7	2.2 ± 0.5	0.6 ± 0.2	0.5 ± 0.3	1.4 ± 0.1
20:3	1.4 ± 0.4	Trace	0.3 ± 0.1	Trace	0.5 ± 0.3	1.7 ± 0.8	1.4 ± 0.7	2.5 ± 0.4
20:4	1.9 ± 0.3	0.3 ± 0.1	2.1 ± 0.2	2.0 ± 0.1	1.6 ± 0.4	7.3 ± 4.9	2.0 ± 1.2	4.4 ± 1.1
20:5	0.1 ± 0.2	0.1 ± 0.2	0.1 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	1.0 ± 0.4	0.2 ± 0.0	0.2 ± 0.1
21:0	2.9 ± 1.6	2.6 ± 0.2	0.3 ± 0.2	2.0 ± 0.9	0.5 ± 0.4	5.5 ± 0.8	1.1 ± 0.2	1.8 ± 0.5
22:0	1.8 ± 0.1	20.0 ± 1.1	0.1 ± 0.0	1.0 ± 0.0	2.2 ± 0.2	ND ^b	2.3 ± 0.3	1.6 ± 0.2
22:1	4.7 ± 1.0	Trace	0.1 ± 0.0	6.1 ± 2.6	10.7 ± 1.6	5.6 ± 2.2	2.8 ± 1.4	4.7 ± 0.8
22:4	0.4 ± 0.1	Trace	0.2 ± 0.2	Trace	0.2 ± 0.1	6.2 ± 1.6	0.5 ± 0.1	4.2 ± 0.9
22:5	Trace	0.8 ± 0.4	0.1 ± 0.5	Trace	Trace	2.5 ± 0.7	0.1 ± 0.0	Trace
23:0	7.6 ± 1.1	Trace	Trace	ND	Trace	1.3 ± 0.3	1.0 ± 0.5	0.7 ± 0.3
24:0	2.0 ± 0.4	26.8 ± 2.9	0.2 ± 0.1	Trace	1.2 ± 0.4	0.2 ± 0.2	0.5 ± 0.2	2.2 ± 0.1
24:1	0.7 ± 0.1	8.8 ± 3.3	0.3 ± 0.2	0.7 ± 0.5	2.5 ± 0.1	2.4 ± 0.4	0.9 ± 0.4	3.2 ± 1.1
Others ^c	3.2 ± 5.5	1.1 ± 3.6	7.8 ± 4.7	0.9 ± 2.1	1.5 ± 2.6	1.6 ± 3.8	2.0 ± 1.2	6.6 ± 2.3
% Sat ^d	81.5	85.8	76.7	82.1	73.8	61.2	71.3	59.4
S/U ^e	5.544	6.600	5.079	4.612	2.754	1.440	2.546	1.948

^a Except as noted, values are means ± standard errors ($n = 6$).

^b ND, not determined.

^c Sum of other minor fatty acids present in the chromatograms.

^d Sum of saturated fatty acids.

^e S/U, saturation index (sum of saturated fatty acids/sum of unsaturated fatty acids).

[vol/vol]) as the solvent system. After visualization of lipid bands with nondestructive I_2 vapor, the bands were scraped off, and material was eluted from the silica gel with $CHCl_3$ -methanol (1:2 [vol/vol]). The material eluted from the band containing both PI and phosphatidylserine (PS) was subjected to TLC on aluminum-backed plates coated with 250 μ m of silica gel G, and these two classes were resolved by using the same solvent system as that described above. The material eluted from the band containing both PE and phosphatidylglycerol (PG) was subjected to TLC on aluminum-backed TLC plates with $CHCl_3$ -methanol- H_2O (65:25:4 [vol/vol]) as the solvent system. All other major classes of phospholipids were well separated and were purified during the first TLC separation.

Preparation of FAME and GLC analysis. Diglycerides of the total polar lipid fraction and of individual phospholipids were first prepared by phospholipase C digestion (10). The diglycerides were saponified with 0.6 N KOH in 90% methanol for 1 h at 65°C (16). After cooling, the glyceryl ethers were removed by extraction with petroleum ether, and then the mixture was acidified with phosphoric acid. The free fatty acids were then extracted into petroleum ether. After the fatty acid extract was dried under N_2 , methyl ester derivatives were prepared by reaction with 0.6 methanolic HCl at 65°C for 1 h (16). The FAME were recovered by extraction with petroleum ether, dried, and then redissolved in hexane for analysis by GLC. Alternatively, ester-linked and amide-linked fatty acids were hydrolyzed by the acidic hydrolysis micromethod of MacGee and Williams (17), in which fatty acids are extracted into hexane and then into a small volume (10 μ l) of trimethyl(α - α -trifluoro- m -tolyl)ammonium hydroxide (TMTFTH [Met-Prep I; Sigma Chemical Co., St. Louis, Mo.]). Fatty acids were converted to their methyl ester derivatives on the column by coinjection with methyl acetate (17).

The FAME profiles and quantitations were done on a Hewlett-Packard 5890 gas chromatograph series II (Hewlett-Packard, Palo Alto, Calif.) equipped with a fused silica capillary column (30 m by 0.32-mm inside diameter) coated with 0.25 μ m of DB-1 (J & W Scientific, Folsom, Calif.) and a flame ionization detector. The oven temperature was programmed at an initial temperature of 180°C for 22 min, which was increased at 10°C/min to 220°C. The final temperature was maintained for 35 min. Both the injector and flame ionization detector temperatures were 250°C; the He flow rate was 1 ml/min. A 50:1 split ratio was used for analyses of approximately 2 μ g of each sample that was injected. Alternatively, FAME were quantified by isothermal analyses at 180°C on a Hewlett-Packard 5792A gas chromatograph equipped with a fused silica capillary column (30 m by 0.32-mm inside diameter) coated with 0.20 μ m of SP2330 (Supelco Inc., Bellefonte, Pa.).

Statistics. The number of determinations represent the values obtained with

individual samples which were obtained from individual rats (both lungs) or the population of *P. carinii* organisms isolated from individual *P. carinii*-infected rats. When replicate GLC analyses of single-lipid samples were performed, the values were averaged to give the value for a single determination. In some cases, single values were obtained by pooling the lipid fractions from several rat lungs or *P. carinii* preparations. Differences in values taken as statistically significant were those at $P < 0.05$ by Student's t test.

RESULTS

Fatty acid profiles of the polar lipid fraction. The total polar lipid fraction includes several minor lipids such as ceramides and glycolipids (e.g., gangliosides [25]), in addition to the major phospholipids that were individually isolated in this study. As expected, because lung surfactant is highly enriched in disaturated PC, the fatty acids of the total polar fraction and of the individual major classes of lung controls had high saturation indices (Tables 1 and 2). To determine whether immunosuppression with methylprednisolone caused changes in fatty acid profiles, the total polar lipid fractions of lungs from normal and immunosuppressed uninfected rats were compared. Immunosuppression apparently caused a relative increase in palmitic acid, the major fatty acid of whole-lung lipids. Consequently, the sum of the percentages of saturated fatty acids in the immunosuppressed uninfected rat samples was greater than that in the normal rat samples. This was reflected in a greater saturation index value in the analysis of the fatty acid profiles of the total polar lipid fraction from whole lungs of immunosuppressed rats.

The fatty acid compositions of the total polar lipid fraction of *P. carinii* organisms differed considerably from those of whole rat lungs (Table 1). Palmitate (16:0) was present in lower concentrations; oleate (18:1) and several other unsaturated acids were present in higher concentrations. The total

TABLE 3. Fatty acid compositions of the major phospholipid classes of whole lungs from immunosuppressed uninfected rats

Fatty acid	Wt % of fatty acids from ^a :							
	LPC	SM	PC	PI	PS	PE	PG	CL
14:0	1.1 ± 0.6	1.0 ± 0.4	1.4 ± 0.6	1.8 ± 0.2	0.8 ± 0.1	3.8 ± 0.6	2.8 ± 0.6	2.6 ± 0.2
15:0	1.2 ± 0.3	1.2 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	0.8 ± 0.3	4.8 ± 0.7	1.0 ± 0.3	3.0 ± 0.2
16:0	36.8 ± 2.7	19.8 ± 2.5	67.0 ± 0.8	51.5 ± 5.8	32.1 ± 5.5	22.7 ± 4.3	57.2 ± 5.2	14.3 ± 4.2
16:1	3.8 ± 0.2	0.6 ± 0.1	3.6 ± 1.0	2.5 ± 1.0	1.7 ± 0.4	2.4 ± 1.0	2.1 ± 0.4	1.5 ± 0.6
17:0	1.6 ± 0.3	0.7 ± 0.2	0.3 ± 0.1	1.0 ± 0.3	0.7 ± 0.1	0.2 ± 0.1	0.7 ± 0.1	8.3 ± 0.8
18:0	24.5 ± 2.7	10.0 ± 1.4	6.7 ± 1.5	19.3 ± 2.4	22.5 ± 5.7	11.8 ± 2.2	8.8 ± 0.9	25.8 ± 5.9
18:1	3.2 ± 1.9	1.9 ± 0.7	4.9 ± 0.8	9.7 ± 1.0	5.4 ± 0.8	13.6 ± 3.3	8.3 ± 2.8	14.8 ± 4.0
18:2	1.1 ± 0.3	0.3 ± 0.1	3.7 ± 0.6	4.5 ± 1.1	4.0 ± 0.4	1.5 ± 0.2	1.0 ± 0.4	5.2 ± 1.5
20:0	1.8 ± 0.3	8.3 ± 2.4	0.3 ± 0.2	1.4 ± 0.4	2.7 ± 0.7	5.3 ± 0.5	1.8 ± 0.4	1.9 ± 0.2
20:3	0.8 ± 0.1	Trace	0.1 ± 0.0	0.8 ± 0.6	2.7 ± 1.3	1.5 ± 1.1	2.1 ± 0.4	0.6 ± 0.4
20:4	0.7 ± 0.1	0.5 ± 0.2	1.0 ± 0.6	2.2 ± 0.9	4.4 ± 1.8	3.1 ± 0.4	0.6 ± 0.2	2.2 ± 0.2
20:5	0.1 ± 0.1	0.4 ± 0.2	0.1 ± 0.0	0.5 ± 0.1	0.8 ± 0.2	0.3 ± 0.2	0.4 ± 0.1	0.7 ± 0.3
21:0	3.9 ± 1.5	0.3 ± 0.1	0.2 ± 0.0	1.0 ± 0.6	2.4 ± 0.4	4.8 ± 0.7	2.8 ± 0.5	3.8 ± 1.4
22:0	1.9 ± 0.2	17.6 ± 3.8	0.4 ± 0.2	0.4 ± 1.2	3.1 ± 0.4	2.2 ± 0.4	1.5 ± 0.2	2.1 ± 0.4
22:1	5.3 ± 1.2	Trace	0.1 ± 0.3	3.0 ± 0.4	10.0 ± 1.4	3.4 ± 1.1	3.6 ± 1.1	2.6 ± 0.5
22:4	0.9 ± 0.1	Trace	0.2 ± 0.1	0.4 ± 0.3	0.7 ± 0.2	14.1 ± 2.4	0.6 ± 0.4	7.5 ± 1.0
22:5	Trace	Trace	ND ^b	0.2 ± 0.5	Trace	Trace	0.1 ± 0.1	Trace
23:0	3.8 ± 1.8	1.9 ± 0.1	ND	0.2 ± 0.1	ND	ND	0.6 ± 0.2	0.4 ± 0.2
24:0	1.4 ± 1.3	26.9 ± 4.0	0.1 ± 0.1	0.7 ± 0.9	Trace	2.0 ± 0.3	0.9 ± 0.2	4.5 ± 1.0
24:1	2.3 ± 1.4	2.7 ± 0.2	Trace	Trace	Trace	0.1 ± 0.1	0.8 ± 0.2	1.6 ± 0.1
Others ^c	5.5 ± 1.1	7.4 ± 2.8	9.0 ± 3.7	0.6 ± 4.2	8.2 ± 2.7	2.1 ± 1.9	5.0 ± 0.8	0.9 ± 3.8
% Sat ^d	78.0	87.7	76.8	78.1	65.1	57.6	78.1	66.7
S/U ^e	4.286	13.703	5.606	3.282	2.192	1.440	3.985	1.817

^a Except as noted, values are means ± standard errors ($n = 6$). Comparisons of the fatty acid compositions of individual phospholipid classes of lungs from normal (Table 2) and immunosuppressed (Table 3) rats were subjected to the Student's t test. Statistically significant differences were observed in the following phospholipids, which are indicated by fatty acids (with the P value given in parentheses): LPC, 16:0 (<0.05), 16:1 (<0.0001), 23:0 (<0.05); SM, 20:0 (<0.05); PC, 18:1 (<0.02), 18:2 (<0.0004); PI, 16:0 (<0.001), 18:0 (<0.002), 18:1 (<0.01); PS, 18:1 (<0.05); CL, 16:0 (<0.05).

^b ND, not determined.

^c Sum of other minor fatty acids present in the chromatograms.

^d Sum of saturated fatty acids.

^e S/U, saturation index (sum of saturated fatty acids/sum of unsaturated fatty acids).

polar lipid fraction of *P. carinii* contained more unsaturated fatty acids than saturated species, as indicated by a saturation index of <1.

Fatty acid compositions of the major glycerophospholipid classes. The saturation indices of the major glycerophospholipids of lungs from normal or immunosuppressed rats were all >1, indicating the presence of more saturated than unsaturated fatty acids (Tables 2 and 3). Individual phosphoglycerides of whole lungs from normal (Table 2) and methylprednisolone-immunosuppressed (Table 3) rats contained the same fatty acids. However, in the lungs of methylprednisolone-treated rats, 16:0 concentrations were elevated in LPC ($P < 0.05$) and PI ($P < 0.001$) and reduced in cardiolipin (CL) ($P < 0.05$), a lipid that is present in low concentrations in lung lipids (1, 9). Other significant differences resulting from immunosuppression are indicated in the footnotes to Table 3.

Compared with the rat lung glycerophospholipids, those of *P. carinii* organisms had higher concentrations of unsaturated acids (lower saturation indices) in the major phospholipid classes PC, PE, and PI and in some minor classes such as PG (Table 4). Compared with the lung lipids of immunosuppressed rats, the organism had more oleic acid (18:1) and less palmitic acid (16:0) in several of its phospholipids. PE of *P. carinii* was particularly distinct from PE of immunosuppressed rat lung lipids. The PE in the organism contained 37% oleate, 18.5% polyunsaturated fatty acids, and only 8% palmitate. The saturation index of *P. carinii*'s PE was <1, the lowest among the major glycerophospholipids analyzed.

The fatty acid compositions of *P. carinii* and the lungs of immunosuppressed uninfected rats were compared, and a

number of significant differences were observed. These are detailed in the footnote to Table 4.

SM. Unlike the fatty acids of glycerolipids, those in sphingolipids are covalently linked to long-chain bases through an amide bond, which is alkali stable. Sphingomyelin (SM) of rat lungs and *P. carinii* was characterized by the abundance of saturated acids 16:0 and 18:0 and especially 22:0 and 24:0 (Tables 2 to 4). Immunosuppression did not appear to affect the SM fatty acid composition of rat lung lipids to a notable degree. The SM fatty acid profile of *P. carinii* was not different from those of both types of rat lung controls.

DISCUSSION

Effects of methylprednisolone on rat lung phospholipid fatty acids. In this study, we observed a relative increase in the amount of 16:0 in the total polar lipid fraction of lung lipids in response to methylprednisolone treatment. This may be accounted for by the following observations. The phospholipids LPC and PI contained increased concentrations of 16:0 in response to methylprednisolone treatment. Also, changes in phospholipid class composition (8) contributed to this dramatic increase in the amount of 16:0 in the total polar lipid fatty acids. In the lungs of methylprednisolone-treated rats the level of PE, the phospholipid with the lowest percentage of 16:0 and total saturated fatty acids, was previously shown to be significantly reduced ($P < 0.01$; $P < 0.0025$), and the levels of LPC ($P < 0.005$) and PI ($P < 0.01$; $P < 0.015$) were elevated (8).

There apparently is tight regulation in the fatty acylation of

TABLE 4. Fatty acid compositions of the major phospholipid classes of *P. carinii carinii*

Fatty acid	Wt % of fatty acids from ^a :							
	LPC	SM	PC	PI	PS	PE	PG	CL
14:0	1.3 ± 0.4	1.2 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	2.3 ± 0.1	3.3 ± 0.5	3.6 ± 0.7	3.6 ± 0.1
15:0	1.3 ± 0.4	0.5 ± 0.3	0.4 ± 0.1	0.9 ± 0.2	5.1 ± 0.8	3.5 ± 1.0	4.0 ± 1.6	5.3 ± 0.1
16:0	48.1 ± 6.4	15.1 ± 4.3	49.1 ± 6.9	17.2 ± 3.3	17.8 ± 3.3	8.0 ± 2.8	28.1 ± 3.5	14.5 ± 4.6
16:1	4.4 ± 1.1	1.0 ± 0.2	1.4 ± 0.3	0.8 ± 0.1	1.9 ± 0.4	2.8 ± 2.1	1.3 ± 0.4	3.4 ± 0.6
17:0	1.0 ± 0.2	0.3 ± 0.1	0.1 ± 0.1	0.6 ± 0.1	8.3 ± 1.5	0.9 ± 0.5	5.0 ± 1.6	1.7 ± 0.2
18:0	14.5 ± 0.6	13.3 ± 4.4	8.8 ± 1.1	26.3 ± 2.1	16.1 ± 1.2	14.4 ± 1.2	18.0 ± 2.1	18.6 ± 2.4
18:1	6.8 ± 1.3	1.4 ± 0.4	34.2 ± 3.6	27.2 ± 0.3	6.5 ± 1.3	36.7 ± 6.3	21.7 ± 0.3	9.8 ± 1.0
18:2	2.4 ± 1.1	0.6 ± 0.2	4.9 ± 0.6	3.1 ± 0.2	Trace	5.3 ± 0.7	4.1 ± 0.2	9.1 ± 0.3
19:0	ND ^b	ND	ND	ND	9.0 ± 3.2	ND	ND	ND
20:0	1.8 ± 0.7	6.7 ± 1.7	0.3 ± 0.1	1.6 ± 0.2	5.0 ± 2.7	1.8 ± 0.9	1.5 ± 0.1	1.7 ± 0.2
20:3	0.3 ± 0.4	Trace	0.2 ± 0.1	0.3 ± 0.1	1.4 ± 1.0	3.6 ± 0.4	6.7 ± 1.4	6.0 ± 0.9
20:4	1.1 ± 0.2	2.9 ± 1.2	4.0 ± 0.4	1.3 ± 0.5	5.7 ± 2.2	1.0 ± 0.4	4.3 ± 1.7	1.8 ± 0.2
20:5	ND	0.3 ± 0.5	0.6 ± 0.1	0.6 ± 0.2	0.4 ± 0.2	0.8 ± 0.4	1.4 ± 0.7	ND
21:0	1.5 ± 0.3	Trace	0.3 ± 0.1	2.0 ± 0.2	11.2 ± 3.9	4.6 ± 1.2	1.6 ± 0.1	3.9 ± 0.3
22:0	1.8 ± 0.4	16.7 ± 3.1	0.4 ± 0.2	2.3 ± 0.1	2.0 ± 0.1	1.1 ± 0.3	1.1 ± 0.5	2.7 ± 0.5
22:1	4.1 ± 1.2	0.8 ± 0.6	Trace	2.1 ± 0.4	7.5 ± 0.2	3.0 ± 1.8	2.7 ± 0.5	6.7 ± 1.0
22:4	1.1 ± 0.3	ND	Trace	1.2 ± 0.5	Trace	7.1 ± 2.3	ND	1.6 ± 1.1
22:5	ND	ND	0.6 ± 0.2	Trace	Trace	0.7 ± 0.2	0.3 ± 0.2	ND
23:0	2.3 ± 1.0	ND	ND	2.1 ± 0.3	1.7 ± 0.5	ND	ND	0.3 ± 0.2
24:0	1.3 ± 0.3	37.3 ± 5.7	0.7 ± 0.3	Trace	2.9 ± 1.0	1.1 ± 0.4	3.2 ± 0.4	0.4 ± 0.1
24:1	2.3 ± 0.2	5.4 ± 0.2	0.4 ± 0.3	1.0 ± 0.3	4.6 ± 0.3	1.5 ± 0.7	0.8 ± 0.7	0.9 ± 0.2
Others ^c	3.7 ± 2.2	2.3 ± 0.9	2.2 ± 2.6	3.4 ± 4.7	0.7 ± 1.4	3.3 ± 1.0	1.4 ± 3.5	7.2 ± 3.3
% Sat ^d	74.9	91.1	61.0	53.7	81.4	38.7	66.1	52.7
S/U ^e	3.329	7.347	1.317	1.428	2.907	0.619	1.527	1.341

^a Except as noted, values are means ± standard errors (n = 6). Comparisons of the fatty acid compositions of individual phospholipid classes of lungs from immunosuppressed uninfected rats (Table 3) and *P. carinii* (this table) were subjected to the Students' *t* test. Statistically significant differences were observed in the following phospholipids, which are indicated by fatty acids (with the *P* value given in parentheses): LPC, 18:0 (<0.002); PC, 16:0 (<0.015), 18:1 (<0.0001), 20:4 (<0.001); PI, 16:0 (<0.0008), 18:0 (<0.025), 18:1 (<0.0001), 18:2 (<0.002); PS, 16:0 (<0.025), 21:0 (<0.025); PE, 16:0 (<0.008), 18:1 (<0.005), 18:2 (<0.0001), 20:4 (<0.002); PG, 16:0 (<0.0005), 18:0 (<0.001), 18:1 (<0.0005), 20:4 (<0.02).

^b ND, not determined.

^c Sum of other minor fatty acids present in the chromatograms.

^d Sum of all saturated fatty acids.

^e S/U, saturation index (sum of saturated fatty acids/sum of unsaturated fatty acids).

lung phospholipids, which was suggested by the data obtained in this study. An interesting relationship was observed in the changes that occurred in the predominant saturated fatty acids, 16:0 and 18:0, as a consequence of immunosuppression. In SM, PC, PS, and PG, large changes in these fatty acids did not occur. However, when major increases in 16:0 occurred (in PI, PE, and LPC), there were concomitant large decreases in 18:0. Therefore, the resultant saturation indices were held relatively constant. Furthermore, the amount of 16:0 was reduced in mitochondrion-specific CL, but in this case, the amount of 18:0 increased. Thus, it appears that 16:0 plus 18:0 concentrations in at least some phospholipids are controlled and kept fairly constant in lung lipids.

PC and LPC. At least all of the C-1 or all of the C-2 positions of PC present in normal rat lungs must be occupied by 16:0 (66% 16:0 in PC [Table 2]). LPC is the most abundant lyso compound in the lung, which is not surprising, since the major lung lipid is PC. LPC, which makes up 1 to 3% of normal rat lung phospholipids (8), can be a precursor in PC biosynthesis or can result from the removal of one fatty acid by phospholipase A action on PC. The concentration of 18:0 LPC molecular species in normal rat lungs was strikingly high. If all PC molecules in normal rat lungs have at least one position on the glycerol backbone occupied by 16:0, the large amount of 18:0 LPC may have resulted from the specific enzymatic hydrolysis at sites occupied by 16:0 (reduced percentage of 16:0 in LPC). Alternatively, much of the LPC may represent normal end products of biosynthetic activity in the lung, in which a third

become acylated with 16:0 (30% 16:0 LPC) and another third become acylated with 18:0 (33% 18:0 LPC).

Upon immunosuppression, an increase in LPC was observed in whole-lung lipids (8). In the present study, data about the molecular species of LPC were obtained. In the lungs of immunosuppressed rats, there were an increase in the amount of 16:0 LPC and a reduction in the amount of 18:0 LPC. Thus, the increased concentration of 16:0 LPC molecular species in the lungs of immunosuppressed rats (possibly in secreted lung surfactant material) could become available to the organism as the result of methylprednisolone treatment. The molecular species profiles of LPC in the lungs of immunosuppressed uninfected rats and in *P. carinii* are similar, and *P. carinii* contained higher concentrations of LPC than both lung controls (8, 20). It is not known whether *P. carinii* (i) metabolizes LPC to form the specific PC molecular species it requires, (ii) biosynthesizes other phospholipids by the addition of another hydrocarbon moiety and head group conversion, or (iii) hydrolyzes the fatty acid and uses it for ATP production. Together, the phospholipid (8) and the fatty acid compositional data in the present report suggest that the organism scavenges LPC from the lung alveolus, concentrates it, and alters it to suit the metabolic needs of the organism.

Phospholipid fatty acids of *P. carinii carinii*. Quantitative differences between the fatty acid profiles of individual phospholipid classes of *P. carinii* and lungs from immunosuppressed rats were evident. The total cellular fatty acid composition of *P. carinii* isolated from rat lungs and placed under short-term

cocultivation conditions with mammalian cells was earlier reported by Paulsrud et al. (19). The fatty acid composition was similar, but not identical, to that of the total lipids extracted from organisms freshly isolated and purified from rat lungs (10a, 11, 14). Among several changes in the total cellular fatty acid profile, Paulsrud et al. (19) observed a relative increase in the amount of 18:1 from 18.5% at day 1 to 31.1% at day 7. It would be interesting to see whether fatty acid compositional changes occur in the polar lipid fraction of organisms analyzed at different times after introduction to short-term culture conditions.

In another study of *P. carinii*, the uptake of a number of different complex fluorescent phospholipids was observed (24). Efficient uptake into trophozoites as well as into cysts, including intracystic bodies, occurred, and only low levels of metabolism of the fluorescent lipids were detected. Thus, the organism may scavenge host fatty acids (14, 18), obtain fatty acids from scavenged host complex lipids, and/or de novo synthesize some of its own fatty acids. De novo fatty acid synthesis has yet to be fully examined with *P. carinii* (14), but the organism apparently can incorporate fatty acids into some phospholipid molecules that it does synthesize. That *P. carinii* synthesizes phospholipids was demonstrated by the incorporation of radiolabeled head group precursors into its phospholipids (5, 20). These phospholipids are probably de novo synthesized or tailored to fulfill the requirements for optimal functioning of its membranes and hence have fatty acids unlike those of lung tissue.

SM. The fatty acid profiles of SM of lungs from normal and immunosuppressed rats were not different, and the fatty acids of *P. carinii*'s SM were similar to those of lung controls. Thus, it appears that host SM molecular species suffice for the proper functioning of the organism's membranes. It is interesting that SM synthesis has been shown to increase in *P. carinii*-infected rat lungs (22), and SM levels in lung surfactant are elevated during PCP (26). Our fatty acid composition data support the earlier observation that *P. carinii* de novo synthesizes ceramides but does not synthesize SM under nonproliferating in vitro conditions (5). The increase in lung surfactant SM in response to PCP probably provides sufficient amounts of this lipid for the pathogen. Together, these observations indicate that the increase in SM associated with PCP results only from increased SM synthesized by lung cells; synthesis or release of SM by the organism is probably not involved.

The detailed characterization of fatty acid compositions of rat lung phospholipids obtained in this study represents the most comprehensive analyses to date. Also, the effects of methylprednisolone immunosuppression on these lipids were identified. This is the first report about the fatty acid compositions of individual phospholipids of the opportunistic pathogen *P. carinii*.

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REFERENCES

- Baxter, C. F., G. Rouser, and G. Simon. 1969. Variations among vertebrates of lung phospholipid class composition. *Lipids* 4:243-244.

- Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911-917.
- Boylan, C. J., and W. L. Current. 1992. Improved model of *Pneumocystis carinii* pneumonia: induced laboratory infections in *Pneumocystis*-free animals. *Infect. Immun.* 60:1589-1597.
- Ellis, J. E., M. Reilly, and E. S. Kaneshiro. 1994. Detection and characterization of an epoxy fatty acid in *Pneumocystis carinii* lipids. *J. Eukaryot. Microbiol.* 41:87S.
- Florin-Christensen, M., J. Florin-Christensen, and E. S. Kaneshiro. 1995. Uptake and metabolism of L-serine by *Pneumocystis carinii carinii*. *J. Eukaryot. Microbiol.* 42:669-675.
- Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* 226:497-509.
- Furlong, S. T., J. A. Samia, R. M. Rose, and J. A. Fishman. 1994. Phytosterols are present in *Pneumocystis carinii*. *Antimicrob. Agents Chemother.* 38:2534-2540.
- Guo, Z., and E. S. Kaneshiro. 1995. Phospholipid composition of *Pneumocystis carinii carinii* and effects of methylprednisolone immunosuppression on rat lung lipids. *Infect. Immun.* 63:1286-1290.
- Harwood, J. L. 1987. Lung surfactant. *Prog. Lipid Res.* 26:211-256.
- Kaneshiro, E. S. 1980. Positional distribution of fatty acids in the major glycerophospholipids of *Paramecium tetraurelia*. *J. Lipid Res.* 21:559-570.
- Kaneshiro, E. S., et al. Submitted for publication.
- Kaneshiro, E. S., M. T. Cushion, P. D. Walzer, and K. Jayasimhulu. 1989. Analyses of *Pneumocystis* fatty acids. *J. Protozool.* 36:69S-72S.
- Kaneshiro, E. S., J. E. Ellis, K. Jayasimhulu, and D. H. Beach. 1994. Evidence for the presence of "metabolic sterols" in *Pneumocystis*: identification and initial characterization of *Pneumocystis carinii* sterols. *J. Eukaryot. Microbiol.* 41:78-85.
- Kaneshiro, E. S., K. B. Meyer, and M. L. Reese. 1983. The neutral lipids of *Paramecium tetraurelia*: changes with culture age and detection of sterol esters in ciliary membranes. *J. Protozool.* 30:392-396.
- Kaneshiro, E. S., and R. G. Sleight. 1994. Biochemistry and metabolism, p. 45-71. In P. D. Walzer (ed.), *Pneumocystis carinii* pneumonia. Marcel Dekker, New York.
- Kaneshiro, E. S., M. A. Wyder, L. H. Zhou, J. E. Ellis, D. R. Voelker, and S. G. Langreth. 1993. Characterization of *Pneumocystis carinii* preparations developed for lipid analysis. *J. Eukaryot. Microbiol.* 40:805-815.
- Kates, M. 1986. Techniques in lipidology. Isolation, analysis and identification of lipids, 2nd ed. Elsevier, Amsterdam.
- MacGee, J., and M. G. Williams. 1981. Preparation of sphingolipid fatty acid methyl esters for determination by gas-liquid chromatography. *J. Chromatogr.* 205:281-288.
- Paulsrud, J. R., and S. F. Queener. 1994. Metabolism of fatty acids and amino acids by cultured *Pneumocystis carinii*. *J. Eukaryot. Microbiol.* 41:633-638.
- Paulsrud, J. R., S. F. Queener, M. S. Bartlett, and J. W. Smith. 1993. Total cellular fatty acid composition of cultured *Pneumocystis carinii*. *J. Clin. Microbiol.* 31:1899-1902.
- Pesanti, E. L. 1987. Phospholipid profile of *Pneumocystis carinii* and its interaction with alveolar type II epithelial cells. *Infect. Immun.* 55:736-774.
- Pneumocystis* Workshop. 1994. Revised nomenclature for *Pneumocystis carinii*. *J. Eukaryot. Microbiol.* 41:121S-122S.
- Rice, W. R., F. M. Singleton, M. J. Linke, and P. D. Walzer. 1993. Regulation of surfactant phosphatidylcholine secretion from alveolar type II cells during *Pneumocystis carinii* pneumonia in the rat. *J. Clin. Invest.* 92:2778-2782.
- Sheehan, P. M., D. C. Stokes, Y.-Y. Yeh, and W. T. Hughes. 1986. Surfactant phospholipids and lavage phospholipase A₂ in experimental *Pneumocystis carinii* pneumonia. *Am. Rev. Respir. Dis.* 134:526-531.
- Sleight, R. G., M. A. Mehta, and E. S. Kaneshiro. 1994. Uptake and metabolism of fluorescent lipid analogs by *Pneumocystis carinii*. *J. Eukaryot. Microbiol.* 41:111S.
- Sorice, M., L. Lenti, R. Misasi, C. Contini, L. Cignarella, T. Griggi, V. Vullo, and C. Masala. 1992. Evidence for the existence of ganglioside molecules on *Pneumocystis carinii* from human lungs. *Parasitology* 105:1-6.
- Su, T. H., V. Natarajan, and W. J. Martin. 1992. Pulmonary surfactant in *Pneumocystis carinii* pneumonia is associated with a marked increase in sphingomyelin. *Am. Rev. Respir. Dis.* 145:A246.
- Telzak, E. E., and D. Armstrong. 1994. Extrapulmonary infection and other unusual manifestations of *Pneumocystis carinii*, p. 361-378. In P. D. Walzer (ed.), *Pneumocystis carinii* pneumonia, 2nd ed. Marcel Dekker, New York.
- Walzer, P. D. 1994. Pathogenic mechanisms, p. 251-265. In P. D. Walzer (ed.), *Pneumocystis carinii* pneumonia, 2nd ed. Marcel Dekker, New York.