Identification and initial characterizations of free, glycosylated, and phosphorylated ceramides of *Paramecium*

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Abstract *Paremecium tetraurelia* contains high concentrations of six ethanolamine sphingolipids in the cell surface membrane surrounding somatic cilia. Three have phosphoryl groups and the other three have phosphonyl groups, and each contains either dihydrosphingosine, sphingosine, or phytosphingosine. In the present study, free, phosphorylated, and three major glycosylated ceramides were identified in the neutral sphingolipid fraction of this organism when it was grown on a crude medium. Individual lipids were characterized by specific staining on thin-layer chromatographic plates and the fatty acids derived from them were identified by gas–liquid chromatography and mass spectrometry. Unlike the esterified fatty acid composition of the ciliate's phospholipids, which have only small amounts of fatty acids greater than 20 carbons in length, the neutral sphingolipids mainly contain fatty acids greater than 22 carbons in length as well as high concentrations of long chain hydroxy fatty acids. The sugars, glucose, galactose, and fructose, were identified in total neutral sphingolipid fraction. The long chain bases in the neutral sphingolipid fraction were mainly C_{18} compounds and were identified as C_{18} dihydrosphingosine, C_{20} sphingosine, and isomers of C_{18} sphingosine. Phytosphingosine was not detected in the neutral sphingolipid fraction obtained from whole cells. Because most of the cell's phytosphingosine-containing ethanolamine sphingolipids are in cilia, the inability to detect phytosphingosine as part of putative precursor ceramide compounds suggests that conversions of the long chain base moiety of ethanolamine phospho- and phosphonosphingolipids occur in situ in the ciliary membrane.—**Kaneshiro, E. S., K. Jayasimhulu, D. Sul, and J. A. Erwin.** Identification and initial characterizations of free, glycosylated, and phosphorylated ceramides of *Paramecium*. *J. Lipid Res.* 1997. **38:** 2399–2410.

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Sphingolipids have been found in nearly all eukaryotes examined (1–6) and have also been detected in bacteria (7). Sphingolipids are important components of biomembrane lipid bilayers, and some are known to be potently bioactive because they play a role in transmembrane signaling, ion translocation functions, or serve as receptors (1–6). Long chain bases (LCB) are formed by the initial condensation reaction involving a fatty-coenzyme A (CoA), commonly palmitoyl-CoA, and serine. The addition of a fatty acid to a LCB via an amide bond forms ceramides, which can serve as precursors for the formation of complex sphingolipids such as phosphosphingolipids and glycosphingolipids (1, 2). The LCB sphingenine (sphingosine) is formed from sphinganine (dihydrosphingosine) by the introduction of a double bond, which occurs at the ceramide (8) or the complex sphingolipid stage in mammals (9). Thus, in mammalian systems, free dihydrosphingosine is apparently not directly converted to free sphingosine, but is desaturated as part of a larger molecule, and is subsequently released upon catabolism (4, 5, 8, 9). Free sphingosine can then be reacylated. Although hydroxysphinganine (phytosphingosine) has been

Abbreviations: DPnE, N-acyl-sphinganine-1-phosphonoethanolamine, N-acyl-dihydrosphingosine-1-phosphonoethanolamine; DPsE, N-acyl-sphinganine-1-phosphoethanolamine, N-acyl-dihydrosphingosine-1-phosphoethanolamine; FAME, fatty acid methyl ester; GLC, gas-liquid chromatography; LCB, long chain base; MS, mass spectrometry; MeOH, methanol; PPnE, N-acyl-D-4-hydroxysphinganine-1-phosphonoethanolamine, N-acyl-phytosphingosine-1-phosphonoethanolamine; PPsE, N-acyl-D-4-hydroxysphinganine-1-phosphoethanolamine, N-acyl-phytosphingosine-1-phosphoethanolamine, SPnE, N-acyl-*trans*-4-sphingenine-1-phosphonoethanolamine, N-acyl-sphingosine-1-phosphonoethanolamine; SPsE, N-acyl-*trans*-4-sphingenine-1-phosphoethanolamine, N-acyl-sphingosine-1-phosphoethanolamine; SS, solvent system; TLC, thin-layer chromatography; TEAE, triethylaminoethyl; TMS trimethylsilyl; WSPG, water-soluble polar group.

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identified as the major LCB in many fungi, protozoans, and plants examined (10–14) and this LCB is present and de novo synthesized in mammals (15–18), its biosynthesis remains poorly understood (11, 15–18).

The ciliated protozoa *Tetrahymena* and *Paramecium* can be grown axenically, and sphingolipids have been examined in these cells (19–31). *Tetrahymena* has ethanolamine sphingophosphonolipids with either hydroxy- or nonhydroxy-fatty acid species (20, 28, 29) and straight- or branch-chained LCB (19). Ceramide-N-monomethylaminoethylphosphonate has also been identified (31). In contrast, six ethanolamine sphingolipids have been identified in *P. tetraurelia*; all have similar fatty acid compositions and contain only straight chain LCB (24). Three were characterized by a stable direct P–C bond (phosphonyl, Pn) whereas the other three had P–O–C (phosphoryl, Ps) bonds. The presence of three different long chain bases further determined their differential migrations on two-dimensional thin-layer chromatographic (TLC) plates. The six were identified as *1*) N-acyl-sphinganine-1-phosphonoethanolamine (N-acyl-dihydrosphingosine-1-phosphonoethanolamine, DPnE), *2*) N-acyl-sphinganine-1-phosphoethanolamine (N-acyl-dihydrosphingosine-1-phosphoethanolamine, DPsE), *3*) N-acyl-*trans*-4-sphingenine-1-phosphonoethanolamine (N-acyl-sphingosine-1-phosphonoethanolamine, SPnE), *4*) N-acyl-*trans*-4-sphingenine-1-phosphoethanolamine (N-acyl-sphingosine-1-phosphoethanolamine, SPsE), *5*) N-acyl-D-4-hydroxysphinganine-1-phosphonoethanolamine (N-acyl-phytosphingosine-1-phosphonoethanolamine, PPnE), and *6*) N-acyl-D-4-hydroxysphinganine-1-phosphoethanolamine (N-acyl-phytosphingosine-1-phosphoethanolamine, PPsE) (24).

Paramecium has been extensively studied to understand the role of the ciliary membrane in controlling swimming behavior. Hundreds of locomotory mutants, some with defective ion channels, have been isolated and characterized by electrophysiologic and other approaches (26, 27). The barium-sensitive locomotory mutant of *P. tetraurelia,* baA, has altered sphingolipid and phosphonolipid compositions, suggesting a role of sphingolipids in the control of ion channels (26). As the ciliary membrane of *Paramecium* is highly enriched in ethanolamine sphingolipids (22–27), these lipids may be required for proper ciliary membrane function.

The neutral sphingolipid fraction of *P. tetraurelia* comprises less than 10% of the total whole cell lipids (23). Glycosphingolipids and important ceramide precursors of complex phosphosphingolipids are found in this fraction. Thus, in the present study we analyzed the neutral sphingolipids to further elucidate the sphingolipid composition in this ciliate. As almost all lipids have been demonstrated to quantitatively change with culture age in axenically grown *P. tetraurelia* (22–25, 32, 33), analyses were also performed on cells at different times after inoculation into fresh culture media. This report represents the first study that identifies the neutral sphingolipids synthesized by this important experimental organism.

MATERIALS AND METHODS

Cells

Mass cultures of *Paramecium tetraurelia* 51s were grown axenically in 500 ml of medium in Fernbach flasks. Cultures were grown for various periods at 25°C, and the cells were harvested by continuous-flow centrifugation as previously described (32). Unless otherwise indicated, ciliates were grown in a crude medium containing stigmasterol, free fatty acids, and animal cephalin (32, 34, 35). In some experiments, cells were grown in a chemically defined synthetic medium (32, 34) which contained the same lipid supplements.

Cilia were isolated from cells by a calcium-shock procedure previously described (32). Briefly, cells were concentrated and washed with distilled water, then suspended in a cold buffered salt solution with sucrose at neutral pH. The cells were then treated with a cold alkaline solution containing glycerol, ethylenediaminetetraacetate, and sucrose, then $CaCl_2$ was added. The deciliated cells were separated from the detached cilia by differential centrifugation. The cilia in the supernate were further purified by passage through sintered glass filters, then the filtrate containing the cilia was washed. Each step in the isolation and purification procedure was monitored by phase microscopy. The purity of the cilia preparation obtained by this protocol has been documented by a number of different microscopic and biochemical criteria (32).

Analyses of culture media

The enriched crude medium, which was used in all of the studies described here, includes proteose peptone, trypticase peptone, Type II-s phosphatidylethanolamine (PsE) from ovine brain, yeast nucleic acids, and other components such as vitamins, salts, and trace metals (32). *Paramecium tetraurelia* will grow in several chemically defined media, but with lowered cell yields. The chemically defined monoolein medium (with reagent grade amino acids, vitamins, and other compounds for which structures are known) contains only stigmasterol and synthetic monoolein as lipid supplements (monoolein defined medium) (32, 34). When cells were grown in the monoolein defined medium, PPnE, PPsE, SPnE, and SPsE, but not DPnE and DPsE, were detected (24).

In the present study, we analyzed both the chemically defined monoolein medium and the enriched crude medium to determine whether sphingolipids were present, and thus could be scavenged from the medium by the organism. Samples (100 ml) were extracted for lipids by biphasic partitioning of compounded media.

After alkaline hydrolysis, LCB and alkali-stable products in the media were recovered by Unisil column chromatography by elution with methanol (MeOH) after the $CHCl_3$-eluting material had been removed. The alkali-stable MeOH fraction was analyzed by TLC and anthrone staining to determine whether glycosphingolipids were detectable. The MeOH fraction was also hydrolyzed under acidic conditions (see below), and the total medium LCB fraction was recovered from Unisil columns. This LCB fraction was analyzed by TLC separation and ninhydrin staining, as described below. The level of LCB detection was at least 0.1 μg, as determined by TLC analyses of authentic LCB standards.

No LCB was detected in the monoolein defined medium. However, dihydrosphingosine and sphingosine, but not phytosphingosine, were detected in the enriched crude medium. Furthermore, we estimated that cells from a 100-ml culture after 5 days of growth in the crude medium contain approximately 0.04 mg of phytosphingosine in PPnE and PPsE alone (22–24, 32). We then added 0.04 mg of authentic phytosphingosine to a 100-ml sample of the crude medium. Phytosphingosine was readily detectable by TLC analysis of the processed enriched crude medium which had been spiked with 0.04 mg of authentic phytosphingosine.

Hence, when *P. tetraurelia* is grown on the chemically defined monoolein medium, it must synthesize all of its LCB. Some dihydrosphingosine and sphingosine could be scavenged from the crude medium by *P. tetraurelia* under that growth condition, but most (if not all) phytosphingosine must be produced by the organism when grown on this medium (10, 11, 14, 15, 18). The ability of *P. tetraurelia* to de novo synthesize LCB is consistent with the demonstration that the LCB moiety of the ethanolamine sphingophospholipids and ethanolamine sphingophosphonolipids was metabolically labeled with radioactive palmitate or serine (25) (D. F. Matesic and E. S. Kaneshiro, unpublished results).

Isolation of neutral sphingolipids

Lipids from whole cells and cilia were extracted with chloroform ($CHCl_3$)–MeOH 2:1 (v/v) or by previously described procedures (22–25, 32, 36). Total lipids were purified by biphasic partitioning (37) and then the solvent volume was reduced and dried under a stream of N_2. The recovered lipids were fractioned by adsorption column chromatography (>90% recovery) using silicic acid (Unisil, Clarkson Chemical Co., Williamsport, PA). After elution of the neutral lipids from the silicic acid columns with $CHCl_3$, the neutral sphingolipids were eluted with $CHCl_3$–MeOH 95:5 (v/v). This neutral sphingolipid fraction was hydrolyzed under mild alkaline conditions using 0.5 N NaOH in $CHCl_3$–MeOH 1:2 (v/v) for 2 h at room temperature, then the products were subjected to Unisil column chromatography. After elution of lipids from the column with $CHCl_3$, the mild alkali-stable fraction was eluted with either $CHCl_3$–MeOH 95:5 (v/v) or MeOH, and designated the neutral sphingolipid fraction. The recovery of alkali-stable lipids was estimated at 90% of the total neutral sphingolipid fraction.

Chromatographic analysis of the neutral sphingolipid fraction

The neutral sphingolipids were characterized by their migrations on aluminum-backed Silica Gel 60 thin-layer chromatographic (TLC) plates (Merck, Darmstadt, Germany) that had been activated by heating at 110°C for 30 min. The plates were developed in either $CHCl_3$–MeOH 9:1 (v/v), solvent system #1 (SS #1) or in $CHCl_3$–MeOH–H_2O 65:25:4 (v/v/v) (SS #2). Lipids were routinely visualized with I_2 vapor, and after sublimation of the I_2, the plates were stained for sugars with either anthrone or orcinol. Individual neutral sphingolipids were purified by preparative TLC. Lipid bands were scraped into funnels lined with Whatman #1 filter paper, and then the individually purified neutral sphingolipids were eluted from the silicic acid with $CHCl_3$–MeOH 1:2 (v/v) followed by MeOH. In some analyses, glycosphingolipids (GSL) were eluted from the TLC silicic acid with $CHCl_3$–MeOH–H_2O 1:1:0.1 (v/v/v). The developed TLC plates were not permitted to completely dry prior to the recovery of the lipids.

Samples of the lipids recovered from the TLC plates were placed in sealed ampoules in MeOH–H_2O–12 N HCl 11:2.6:1 (v/v/v) and heated for 18–24 h at 80°C (24, 36). Fatty acid methyl esters (FAME) were extracted with hexane and the remaining material was evaporated to dryness under N_2. This residue was either directly analyzed or further processed. For further extraction, the residue was redissolved in distilled water, the pH was adjusted to 11.0 with NaOH, and the LCB were extracted with $CHCl_3$. The material remaining after $CHCl_3$ extraction was designated the fraction containing water-soluble polar groups (WSPG). After neutralization of the WSPG fraction, both the LCB and the WSPG fractions were evaporated to dryness under N_2.

Structural identification of LCB in individual LCB fractions or in the combined LCB plus WSPG fractions was done by TLC, gas–liquid chromatography (GLC), and GLC-high resolution mass spectrometry (MS). Silica gel G TLC plates impregnated with 0.1 M Na_2CO_3

were developed in $CHCl_3$–MeOH–NH_4OH 120:30:3 (v/v/v) (SS #3). After development, ammonia was thoroughly evaporated by placing the plates in a fume hood overnight. The LCB on TLC plates were visualized with 0.2% ninhydrin in butanol (38). Alternatively, LCB released by hydrolysis of sphingolipids were acetylated with acetic anhydride, converted to their trimethylsilyl (TMS) derivatives, and analyzed by GLC on 6-ft glass columns packed with 3% OV-17 (24, 25, 36).

The FAME obtained from the acid hydrolysates were also analyzed by GLC using 10% EGSS-X columns as previously described (24, 36). Individual fatty acids were identified by elution times, cochromatography with authentic standards (methyl esters of α-hydroxy stearate, Mix LA 203 and Mix LA 207 from Applied Sciences, State College, PA, and Mix HEM-2 from Supelco, Inc., Bellefonte, PA). Furthermore, the structural identities of individual FAME were verified by MS (see below).

Sugars in the WSPG fractions were analyzed by TLC on aluminum-backed Silica Gel 60 plates developed with acetone–n-butanol–H_2O 50:35:15 (v/v/v; SS #4). Sugars were visualized with orcinol or aniline-diphenylamine-phosphoric acid reagent (38). Sugars were also analyzed by GLC (39). The fractions containing the LCB and WSPG were evaporated to dryness under N_2, and 0.2 ml of N-trimethylsilylimidazole (TMSIM)–N, O-*bis*-(trimethylsilyl) acetamide (BSA)–trimethylchlorosilane (TMCS) 3:3:2 (v/v/v) (Alltech Inc., Deerfield, IL) was added and fractions were allowed to stand for 5 min (39, 40). Aliquots of the resultant trimethylsilyl (TMS) sugar derivatives were analyzed using 3% silicone OV-101 at a column temperature of 190°C. The injection temperature was 250°C, detector temperature was 300°C, and the N_2 carrier gas was set at 20 psi.

Mass spectral analyses

Fatty acid methyl esters were analyzed by GLC–high resolution mass spectrometry (GLC–MS) using a capillary GLC column coated with SE-54. The GLC was interfaced with a mass spectrometer (Kratos MS-80, Kratos, Manchester, U.K.) as previously described (24, 36). Electron impact mass spectral data were processed on a Data General NOVA/4 computer with a DS-55 data system (DS) and individual FAME were identified by their fragmentation patterns, ion masses, and elemental compositions.

Acetylated LCB were converted to their O-TMS derivatives with *bis*(trimethylsilyl)trifluoroacetamide (BSTFA, Applied Sciences). The TMS-derivitized samples were analyzed by GLC–MS–DS using a 30 m, 0.32 mm i.d. SPB-5 capillary column to separate the individual LCB derivatives (24, 36). The identifications of individual LCB were further confirmed by fast atom bombardment MS (FAB–MS) of the underivatized free ceramides as previously described (24, 36).

Chemicals

N-stearoylsphingosine monoglucoside, dihydrosphingosine, sphingosine, phytosphingosine, N-palmitoylsphingosine, and monosaccharide reference compounds were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Effects of culture age

The neutral sphingolipid content of *P. tetraurelia* cells increased with culture age (**Fig. 1**). Stationary phase cells (day-7) were inoculated into 10 times the volume of fresh enriched crude medium. Lag phase lasted for 1–2 days; log phase occurred over days 3–5, then the cultures entered stationary phase. Death phase was not evident until after day 8 (32). The concentration of the total neutral sphingolipid fraction relative to total cellular lipids was lowest in day-1 cells (4.3%) and highest in day-7 cells (9.7%). In contrast to the quantitative changes observed, there were no qualitative differences in the relative amounts of individual neutral sphin-

Fig. 1. Change with culture age in the relative concentration of the total neutral sphingolipid fraction of *P. tetraurelia* lipids obtained from whole cells grown on the enriched crude medium. Values represent means ± SEM of 5 determinations.

golipids extracted from cells of different culture ages. Analysis by TLC of the neutral sphingolipids of day-1, day-3, and day-7 cells showed the presence of the same lipid bands at all 3 days of culture growth. Also, TLC analysis of the neutral sphingolipids obtained from cells grown in the enriched crude medium revealed no qualitative differences between these lipids and those obtained from cells grown in the chemically defined synthetic medium.

Long chain base composition of the total neutral sphingolipid fraction

The ethanolamine sphingophospholipids and sphingophosphonolipids of *Paramecium* contain three LCB: C_{18} dihydrosphingosine, C_{18} sphingosine, and C_{18} phytosphingosine (24). The relative proportions of lipids with these three bases found in whole cells grown on the enriched crude medium changed with culture age, but PPnE and PPsE remained the major components throughout. Interestingly, phytosphingosine was not detected by TLC or GLC analyses of the hydrolysates of the neutral sphingolipids. No phytosphingosine was detected in the total neutral sphingolipid fraction from cells of varying culture age grown on the crude medium, and no phytosphingosine was detected in the neutral sphingolipid fraction obtained from simulated day-1 cells (day-7 cultures diluted 1:1 with fresh medium) grown in either the crude or a chemically defined medium (32). Furthermore, phytosphingosine was not detected in hydrolysates obtained from any of the individual lipids of the neutral sphingolipid fraction. These observations suggest that phytosphingosine was synthesized as part of polar complex sphingolipids, and not via ceramide intermediates.

Analysis by GLC of LCB obtained from hydrolysates of the total neutral sphingolipid fraction of *P. tetraurelia* grown on the crude medium indicated the presence of five major and at least two minor components (**Fig. 2**). Mass spectral analysis of the major GLC peaks demonstrated that one peak contained C_{18} dihydrosphingosine (Fig. 2D), another contained C_{20} sphingosine (Fig. 2F), and three peaks represented isomers of C_{18} sphingosine (Fig. 2 B, C, and E). The molecular ion *m/z* 485 of acetyl ditrimethylsilyl sphingosine was not observed. A peak at *m/z* 470 ($M^{+}\cdot$–CH_3), characteristic of TMS derivatives was observed in spectra of the C_{18} sphingosines. Similarly, an ion at *m/z* 472 ($M^{+}\cdot$–CH_3) was observed in the spectrum for C_{18} dihydrosphingosine. The characteristic base peaks *m/z* 311, *m/z* 313, and *m/z* 339 were present in spectra of the C_{18} sphingosines, C_{18} dihydrosphingosine, and C_{20} sphingosine derivatives, respectively. Although future studies should include detailed characterizations of the C_{18} sphingosine isomers, these analyses were not performed in the present study due to the small amount of LCB present in this fraction.

Relative to the elution time of C_{18} dihydrosphingosine, these C_{18} sphingosines had relative retention times (RRT) of 0.84, 0.93, and 1.23. The LCB that had the longest elution time was identified as C_{20} sphingosine (RRT, 1.50). In addition to these major LCB, two minor peaks observed by GLC were identified by MS as another isomer of C_{18} sphingosine (RRT, 1.23), and another isomer of C_{18} dihydrosphingosine (RRT, 1.30). The LCB isomers with longer elution times may represent those with branched-chains. Branched-chain sphingosines, exhibiting longer GLC elution times than those of straight-chained sphingosines, have been characterized in *Tetrahymena* (19), but these *Paramecium* C_{18} sphingosine isomers were not further characterized in the present study. Although the neutral sphingolipid concentrations in *P. tetraurelia* cells increased, the compositional profile of the LCB in the total neutral sphingolipid fraction did not change with culture age (**Table 1**). The constant LCB composition of this major lipid fraction was unexpected and unusual for this cell system. Most other lipid and lipid components have been shown to change as this ciliate progresses through axenic culture growth in the enriched crude, or chemically defined, media (22–24, 32, 33).

The MS analysis indicated no detectable phytosphingosine was present in the samples of LCB in the total neutral sphingolipid fraction. In these GLC–MS analyses, the five LCB identified were present at mmol levels. If phytosphingosine were present at a level of 1 nmole, it would have been detected by these GLC–MS analyses.

Monosaccharide composition of the total neutral sphingolipid fraction.

Analysis by TLC of the sugars obtained from hydrolysates of the total sphingolipids obtained from whole cells grown on the enriched crude medium revealed the presence of at least three components tentatively identified as fructose, glucose, and galactose. Analysis by GLC indicated the *Paramecium* total neutral sphingolipid fraction sugars cochromatographed with authentic standards of D-fructose, D-galactose, and the α and β anomers of D-glucose. Fructose accounted for 15% of the total, while galactose and glucose were present in approximately equal amounts (**Table 2**). A fifth component was present in the pentose region of the gas chromatogram, and accounted for 12% of the total monosaccharides in that fraction. This component did not chromatograph with authentic standards of arabinose, ribose, or xylose and remains unidentified. Additional structural analyses of the GSL sugars were not performed in the present study.

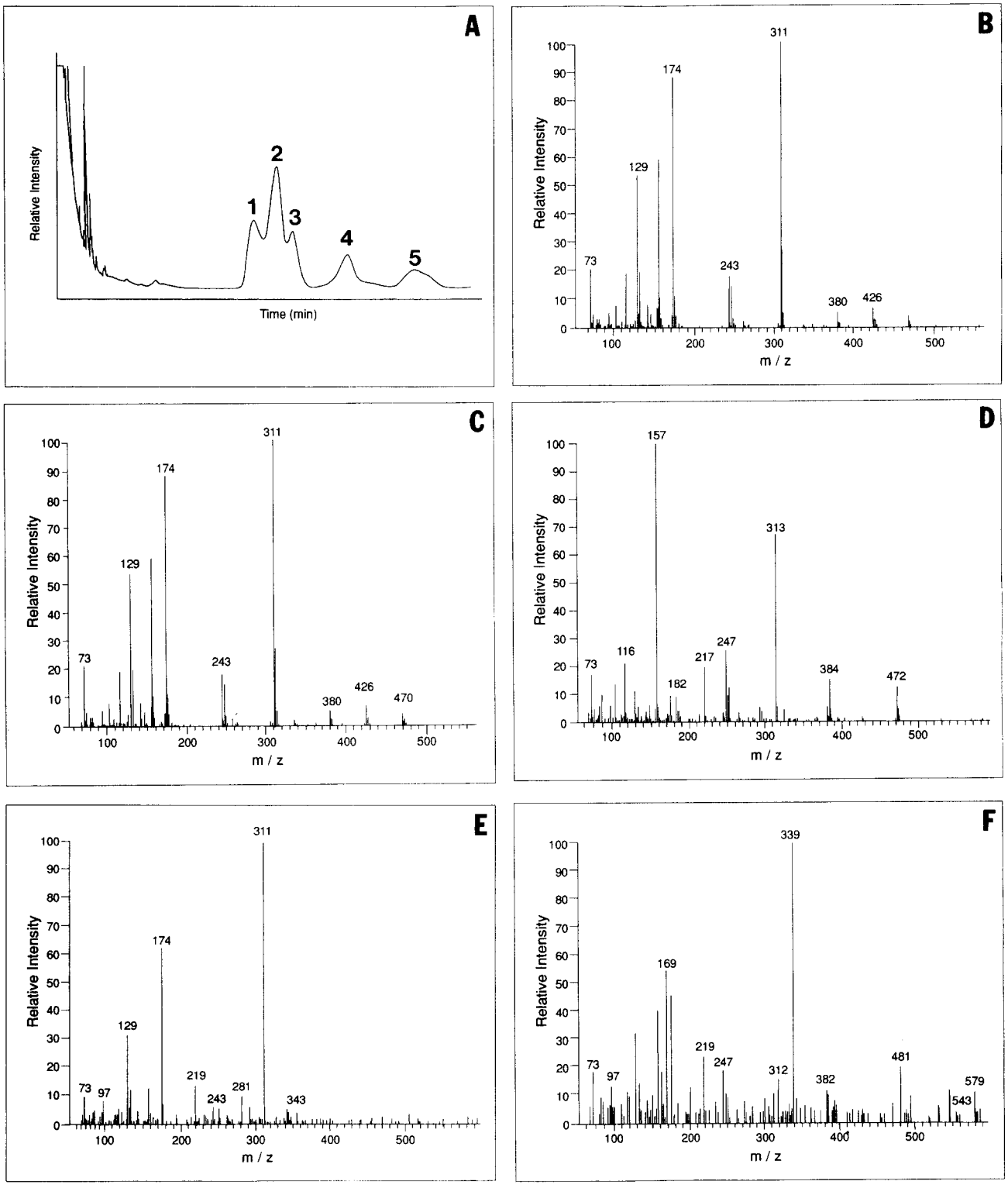

Fig. 2. Mass spectra of LCB in hydrolysates of the neutral sphingolipid fraction obtained from *P. tetraurelia* whole cells grown on the enriched crude medium. **A:** A typical GLC tracing showing the five major LCB in the total neutral sphingolipid fraction. **B:** Mass spectrum of a C_{18} sphingosine, corresponding to peak #1 in the GLC tracing. **C:** Mass spectrum of a C_{18} sphingosine corresponding to GLC peak #2. **D:** Mass spectrum of a C_{18} dihydrosphingosine corresponding to GLC peak #3. **E:** Mass spectrum of a C_{18} sphingosine corresponding to GLC peak #4. **F:** Mass spectrum of a C_{20} sphingosine corresponding to GLC peak #5.

TABLE 1. Long chain base composition of the total neutral sphingolipid fraction of *Paramecium tetraurelia* cells at different culture ages

Long Chain Base	GLC Peak #	Culture Age (days) 1	3	5	7
C_{18} sphingosine	1	20.2 ± 0.8	20.1 ± 0.4	20.4 ± 2.5	20.4 ± 1.1
C_{18} sphingosine	2	38.6 ± 1.4	38.5 ± 0.6	39.2 ± 1.0	38.8 ± 1.2
C_{18} dihydrosphingosine	3	13.9 ± 0.8	14.1 ± 0.2	13.9 ± 0.6	13.8 ± 0.6
C_{18} sphingosine	4	18.0 ± 2.2	15.7 ± 0.8	15.5 ± 0.6	16.2 ± 0.4
C_{20} sphingosine	5	9.4 ± 2.3	11.6 ± 0.3	11.0 ± 1.5	10.9 ± 0.5

The total neutral sphingolipid fraction was isolated from whole cells grown in the enriched crude medium. After acid hydrolysis, the long chain bases of the fraction were acetylated and silylated then quantified by GLC. Weight percent values are means ± SEM of 3–4 determinations. Peaks are numbered according to the order in which they eluted from the GLC column (see Fig. 3).

Fatty acid composition of the total neutral sphingolipid fraction

The FAME extracted from hydrolysates of the total neutral sphingolipid fraction contained some species characteristically found in other lipid fractions, particularly 16:0, 18:0, and 18:1. However, substantial amounts of long chain saturated and monounsaturated fatty acids of C_{22} to C_{26}, as well as α-hydroxy acids, not previously described from *Paramecium*, were detected in this fraction (**Table 3**). The two largest components observed in all gas chromatograms were consistently the peaks that contained 24:1, and 24:0 plus OH–20:0. Branched chain fatty acids were not detected in these lipids, nor have they been found in any other lipids of *P. tetraurelia* (22, 24, 32). The relative amounts of unsaturated fatty acid in the total neutral lipid fraction decreased with culture age. The greatest change in the relative amounts of a single fatty acid was observed in 24:1, which significantly decreased from day-1 to day-7 ($P < 0.05$).

Glycosphingolipids

The neutral sphingolipid fraction (from day-5 cells) contained several TLC components that were detectable by I_2, and stained with anthrone and orcinol, indicating that they were glycosylated. These lipids accounted for the bulk of the neutral sphingolipid fraction (**Fig. 3B**; bands 4, 5, and 7). When the material from the three anthrone- and orcinol-positive bands were recovered from the TLC plates (which had been developed in SS #1), pooled, and the pooled material rechromatographed using SS #2, more than three bands were resolved (Fig. 3B). The three major groups of GSL were designated (from the most mobile in SS #2) as GSL III = band 3 (R_f 0.78); GSL II = band 2 (a single band in SS #1 that resolved into two broad bands in SS #2 with R_f 0.63 and R_f 0.52); and GSL I = band I (R_f 0.23).

For further analysis of the GSL, the neutral sphingolipid fractions from several batches of *P. tetraurelia* whole cells grown on the enriched cruded medium for 3–5 days were pooled, and individual GSL were isolated by TLC using SS #1. A mixture of GSL was recovered, then GSL I, GSL II, and GSL III were purified by preparative TLC using SS #2. Glycosphingolipid II constituted about 90% by weight of the total GSL obtained by preparative TLC. On the TLC plates developed in SS #2, the R_f value of the more mobile component of GSL II was the same as that of authentic N-stearolysphingosine monoglucoside (Fig. 3C). When the LCB fraction obtained from hydrolysates of GSL II was subjected to TLC (using SS #3 for development), only two ninhydrin-positive components were detected. They had an R_f value identical to those of authentic dihydrosphingosine and sphingosine, respectively (**Table 4**). Mass spectral analysis of the LCB fraction obtained from GSL II confirmed the identification of both dihydrosphingosine and sphingosine (Fig. 2B–2F). Trace amounts of a third LCB were detected, but no phytosphingosine was detected.

Analysis by TLC of the WSPG fraction of the TLC-purified major GSL, GSL II, indicated the presence of three components that reacted with aniline-diphenyl

TABLE 2. Monosaccharide composition of the total neutral sphingolipid fraction of *Paramecium tetraurelia* whole cells grown on the enriched crude medium

Monosaccharide	RRT	Relative Concentration
		% of total
D-fructose[a]	0.78	15.61 ± 0.11
D-galactose[a]	0.88	34.63 ± 1.88
D-glucose		37.63 ± 1.06
α Anomer	1.00	
β Anomer	1.20	
Unidentified	0.52	11.95 ± 0.94

Values are means of (2) replicate analyses, ±SD. Relative retention times (RRT) were calculated by the GLC elution times compared to that of the α-D-glucose derivative.

[a]The RRT is of the major anomer peak. The concentration includes the sum of the major and minor anomer peaks.

TABLE 3. Fatty acid composition of the total neutral sphingolipid fraction of *Paramecium tetraurelia* whole cells grown on the enriched crude medium and harvested at different culture ages

Fatty Acid	RRT	Culture Age (days) 1	3	5	7
14:0	0.21	1.1 ± 0.6	1.1 ± 0.6	1.5 ± 0.7	2.4 ± 1.4
16:0	0.48	1.6 ± 0.4	1.5 ± 0.3	2.3 ± 0.8	1.4 ± 0.3
17:0	0.69	0.4 ± 0.1	0.4 ± 0.2	0.6 ± 0.3	0.4 ± 0.2
18:0	1.00	4.1 ± 0.3	3.6 ± 0.3	4.1 ± 0.4	4.1 ± 0.6
18:1	1.11	1.0 ± 0.3	0.9 ± 0.5	1.4 ± 0.7	1.2 ± 0.7
20:0	1.57	0.7 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
22:0 + OH-18:0	2.06	7.6 ± 1.5	8.5 ± 1.3	8.4 ± 1.0	7.4 ± 1.7
22:1	2.15	2.7 ± 0.9	2.8 ± 1.1	2.5 ± 1.0	4.7 ± 1.4
23:0	2.27	2.4 ± 0.2	2.7 ± 0.2	2.5 ± 0.1	2.5 ± 0.2
23:1	2.35	1.3 ± 0.2	1.1 ± 0.2	0.9 ± 0.1	1.4 ± 0.4
24:0 + OH-20:0	2.51	10.2 ± 1.1	11.0 ± 1.4	12.0 ± 1.8	13.7 ± 2.8
24:1	2.58	31.9 ± 3.0	26.9 ± 2.2	26.2 ± 2.0	23.4 ± 2.1
25:0	2.67	1.5 ± 0.1	1.6 ± 0.1	1.0 ± 0.2	1.6 ± 0.1
25:1	2.74	3.8 ± 0.4	3.9 ± 0.2	3.0 ± 0.4	4.5 ± 0.5
26:0 + OH-22:0	2.88	4.0 ± 1.2	4.6 ± 1.6	4.2 ± 1.4	5.0 ± 1.3
26:1	2.94	8.0 ± 1.3	9.2 ± 1.1	8.5 ± 1.4	8.3 ± 0.7
OH-23:0	3.06	2.2 ± 0.3	3.3 ± 0.9	2.3 ± 0.5	1.9 ± 0.3
OH-24:0	3.28	5.9 ± 0.7	6.3 ± 0.6	6.7 ± 1.0	5.0 ± 0.8
OH-24:1	3.42	9.6 ± 1.6	7.4 ± 0.4	7.8 ± 1.1	5.9 ± 1.1
Others[a]		0.0	2.7	3.7	4.7
Sat/Unsat[b]		0.72	0.86	0.91	0.93

The neutral sphingolipid fraction was obtained by silicic acid column chromatography after mild alkaline hydrolysis. The neutral sphingolipid fraction was subjected to acid hydrolysis and methanolysis, and the resulting FAME were extracted and quantified by GLC. Values are means ± SEM of 4–6 determinations. RRt, retention time relative to 18:0.

[a]Sum of individual fatty acid components present in concentrations <0.5 wt %.

[b]Sum of saturated fatty acids/sum of unsaturated fatty acids.

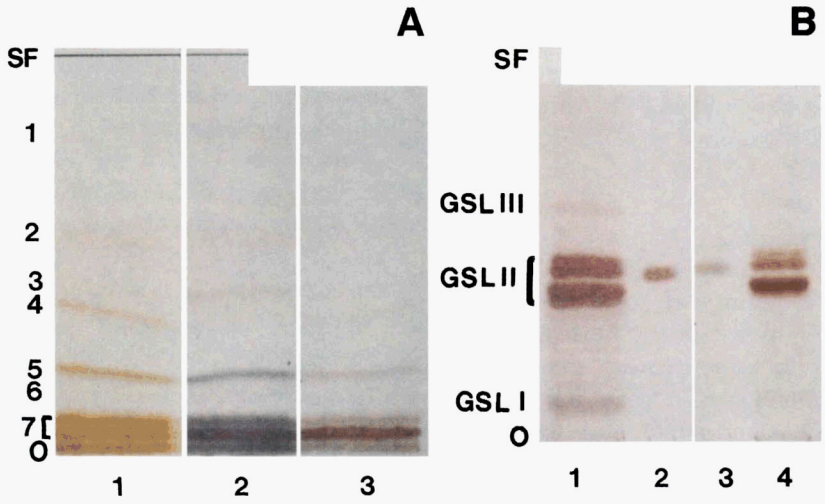

Fig. 3. Thin-layer chromatographic analyses of the neutral sphingolipids of *P. tetraurelia* obtained from whole cells grown on the enriched crude medium. **A:** Total *P. tetraurelia* neutral sphingolipid fraction developed in solvent system #1. Lipids were stained with iodine vapor (lane 1), orcinol (lane 2), and anthrone (lane 3) reagents. Band 7 contains GSL I, band 5 corresponds to GSL II, and band 4 corresponds to GSL III. These three bands stained with all three TLC reagents. **B:** The glycolipid components of the *P. tetraurelia* neutral sphingolipid fraction recovered from preparative TLC plates developed in solvent system #2 and stained with anthrone. Lane 1, sample of pooled GSL. Lands 2 and 3, authentic N-stearoylsphingosine monoglucoside; R_f, 0.63. Lane 4, GSL II (band 5 in Fig. A) isolated by preparative TLC using SS #1, and rechromatographed in SS #2. In SS #2, this band resolved into two components with R_f values of 0.63 and 0.53.

TABLE 4. Identification by TLC analysis of sphingolipids of *P. tetraurelia* whole cells grown on the enriched crude medium

	R_f	Ninhydrin	I_2	Anthrone
LCB standards[a]				
C_{18} sphingosine	0.47	+	+	ND[b]
C_{18} dihydrosphingosine	0.40	+	+	ND
C_{18} phytosphingosine	0.24	+	+	ND
P. tetraurelia LCB[a]				
Total neutral sphingolipid fraction[c]				
#1	0.47	+	+	ND
#2	0.41	+	+	ND
GSL II[c,d]				
#1	0.47	+	+	ND
#2	0.41	+	+	ND
Authentic N-palmitoylsphingosine[e]	0.55	ND	+	–
P. tetraurelia free ceramide[e,f]	0.55	ND	+	–
Unidentified *P. tetraurelia* free ceramide[e]	0.45	ND	+	–
P. tetraurelia phosphoceramide[e]	0.01	ND	+	–

[a]Developed in SS #3.
[b]ND, not determined.
[c]LCB fraction obtained after acid hydrolysis. Only two components were detected.
[d]GSL II was purified by preparative TLC using SS #1, then acid-hydrolyzed.
[e]Developed in SS #1.
[f]The free ceramide fraction was purified by preparative TLC using SS #1, then analyzed on the same plate with authentic N-palmitoylsphingosine.

amine-phosphoric acid. Glucose was tentatively identified as one of three sugar components detected; the others were not further characterized.

In the present study, only one set of samples of individually isolated classes was available for fatty acid analysis. The data obtained suggest that they contain distinct fatty acid compositions, which may contribute to the ability to separate them by chromatographic procedures. Analysis by GLC of FAME obtained from the hydrolysate of GSL II revealed that saturated fatty acids containing 20–26 carbons and their α-hydroxy analogs, 24:1, and 26:1, accounted for approximately 90% of the total fatty acids of this lipid (**Table 5**). The identities of these and all other FAME described in this report were confirmed by high resolution MS. In contrast, 16:0, 18:0, and 18:1 accounted for about 75% of TLC-purified GSL I, while GSL III had a fatty acid composition intermediate between those of GSL I and GSL II (Table 5).

Free and phosphorylated ceramides

Analysis by TLC of the total neutral sphingolipid fraction in SS #1 indicated bands 2 and 3 (Fig. 3A) were visible with I_2 staining, but they did not stain with anthrone (band 3 was visible by orcinol staining, but did not have the same color exhibited by the GSL on

TABLE 5. Fatty acid composition of the major neutral sphingolipids isolated from *Paramecium tetraurelia* whole cells grown on the enriched crude medium

		Glycosphingolipids				
Fatty Acid	RRT	I	II	III	Free Ceramide[a]	Phosphoceramide
14:0	0.21	0.8	trace	trace	0.5	0.8
16:0	0.48	34.3	1.8	17.0	31.0	16.4
16:1	0.56	0.6	trace	1.7	trace	1.0
18:0	1.00	20.4	4.3	11.4	51.5	7.6
18:1	1.11	20.2	0.7	4.7	trace	46.8
18:2	1.31	0.0	0.0	0.0	0.0	9.6
19:0	1.34	5.5	trace	1.5	0.0	0.0
20:0	1.57	0.0	0.0	0.0	0.5	3.8
22:0 + OH-18:0	2.06	9.2	15.0	17.3	0.0	0.0
22:1	2.15	5.6	trace	0.0	3.2	0.7
23:0	2.27	trace	2.5	0.5	trace	1.5
24:0 + OH-20:0	2.51	0.8	10.0	10.3	2.3	1.3
24:1	2.58	trace	29.4	18.0	1.5	trace
25:0	2.67	trace	0.6	trace	0.0	0.0
26:0 + OH-22:0	2.88	trace	6.2	0.6	trace	2.0
26:1	2.94	trace	7.2	4.0	1.3	trace
OH-23:0	3.06	0.5	1.9	trace	0.0	0.0
OH-24:0	3.28	trace	6.9	trace	0.0	0.0
OH-24:1	3.42	0.6	11.1	1.1	0.0	0.0
Others[b]		7.7	2.5	1.6	8.4	8.5
Sat/Unsat[c]		2.65	1.02	1.99	14.30	0.57

Individual lipids obtained by preparative TLC of the neutral sphingolipid fraction were subjected to acid hydrolysis and methanolysis, and then the FAME were extracted and analyzed by GLC. Values are expressed as weight percent, and represent averages of duplicate analyses on single preparations. RRT, retention time relative to 18:0.

[a]The faster migrating free ceramide band corresponding to band 2 in Fig. 3A.
[b]Sum of fatty acids present in concentrations less than 0.5%.
[c]Sum of saturated fatty acids/sum of unsaturated fatty acids.

the plate). One of these lipids (band 2) had an R_f that was identical to that of authentic N-palmitoylsphingosine (Table 4), and was identified as a free ceramide. The second lipid (band 3) had a lower R_f value than the free ceramide (Fig. 3A) and was not further characterized. It cannot be ruled out that band 3 represents dihydroceramide, which would be expected to be more polar than N-palmitoylsphingosine. Another lipid remained near the origin in SS #1, and migrated just below GSL I in SS #2. This anthrone-negative component, which was metabolically radiolabeled with ^{32}P (J. A. Erwin and E. S. Kaneshiro, unpublished results), was tentatively identified as a phosphoceramide.

Unlike the GSL, the free ceramide (band 2 in Fig. 3A) and the phosphoceramide fatty acids did not include substantial amounts of hydroxy acids. The free ceramides contained high concentrations of saturated fatty acids; greater than 80% were either 16:0 or 18:0. In contrast, the phosphoceramides had high concentrations of unsaturated fatty acids, especially 18:1 (Table 5).

Cilia

Only a single orcinol-positive component was detected in TLC analyses of the neutral sphingolipid fraction obtained from *P. tetraurelia* cilia using either SS #1 or SS #2. This band had R_f values similar to that of the faster migrating component (0.09 in SS #1; 0.63 in SS #2) of GSL II and of authentic glucosyl-ι-1-sphingosine (**Fig. 4**).

To determine whether the cilia glucosphingolipid could have originated from the medium by simply inserting into the cell surface membrane surrounding the organelle, the crude medium was analyzed for glycosphingolipids. Two alkali-stable anthrone-positive components were detected in lipids extracted from 100 ml of the enriched crude medium. The R_f values of these medium components in SS #2 were 0.12 and 0.16. Thus, the cilia glycosphingolipid was apparently synthesized by the organism because its TLC migration differed from those of the two glycosphingolipids in the crude medium. Also, if exogenous glycosphingolipids present in the crude medium were simply inserted in to the ciliary membrane, two components would be expected to be present and detectable in the cilia.

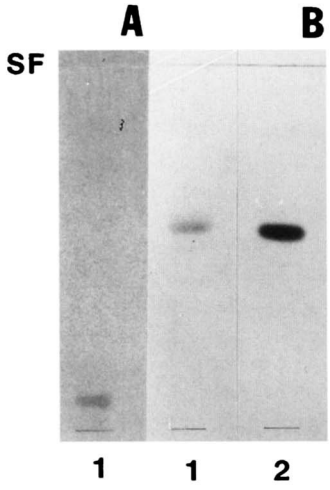

Fig. 4. The neutral sphingolipid fraction obtained from a purified preparation of *P. tetraurelia* cilia isolated from cells grown on the enriched crude medium analyzed by TLC and visualized with orcinol. **A:** Development in SS #1 showed that only one orcinol-positive component was present. **B:** Development in SS #2 showed only one orcinol-positive component. Lane 1, ciliary neutral sphingolipids; lane 2, authentic monoglucosyl-1-sphingosine ceramide.

DISCUSSION

Composition of the neutral sphingolipid fraction

The ethanolamine sphingophospho- and sphingophosphonolipids constitute 3.8% and 15.5% of *P. tetraurelia*'s extractable cellular and cilia lipids, respectively (23). It was estimated that the neutral sphingolipid fraction constitutes about 7% of the total cellular lipids, and 4% of ciliary lipids (23). The present report represents the first study of the neutral sphingolipid fraction of this ciliate; free, phosphorylated, and glycosylated ceramides were identified. Analyses of the fatty acids associated with this fraction also showed that this ciliate contains C_{23}–C_{26} fatty acids as well as α-hydroxy fatty acids, never before demonstrated in *Paramecium* lipids. Unlike *Tetrahymena*, which has hydroxy fatty acids in its ethanolamine sphingophosphonolipids, hydroxy fatty acids were found associated with the GSL in *P. tetraurelia*.

The free ceramide and the phosphoceramides may represent precursors, or catabolic products, of complex ethanolamine sphingophospho- and sphingophosphonolipids. However, only C_{18} dihydrosphingosine, one C_{18} sphingosine isomer, and C_{18} phytosphingosine were detected in *P. tetraurelia*'s ethanolamine sphingophospho- and sphingophosphonolipids (24). Thus the other C_{18} LCB isomers and the C_{20} sphingosine identified in the neutral sphingolipid fraction are probably not involved in the metabolism of complex ethanolamine sphingophospho- or sphingophosphonolipid in this organism.

The fatty acid profiles of the free ceramides (highly saturated) and the phosphoceramides (highly unsaturated) suggest that these are not direct products of phosphorylation–dephosphorylation reactions of the other. However, the similarity between the fatty acid compositions of the free ceramide and the ethanola-

mine sphingophospho- and sphingophosphonolipids, which contained 16:0 and 18:0 as the two most abundant species (23, 24), is consistent with the possibility that the free ceramides are direct precursors (or products) of these complex sphingolipids. The fatty acid compositions of the GSL, characterized by high concentrations of C_{22} and hydroxy fatty acids, suggest that the free ceramides are not direct precursors of the glycosylated ceramides.

It has been reported that direct hydroxylation of dihydrosphingosine occurs in the rat resulting in the formation of complex lipids containing phytosphingosine (15, 17). In the rat experiments, radioactivity from dihydrospingosine was apparently incorporated into phytosphingosine bound as free ceramides (17), hence complex phytosphingosine-containing sphingolipids may be synthesized via ceramide intermediates in the rat. Unlike the situation in rats (17), phytosphingosine was not detected in the free ceramides of *Paramecium*, suggesting that LCB moiety of PPsE and PPnE was formed after the addition of both the fatty acid moiety and the head group. Also, the absence of phytosphingosine in this fraction indicates that it is highly unlikely that catabolism of PPsE or PPnE contributed to the free ceramides in this ciliate.

The absence of C_{20} sphingosine in the ethanolamine sphingolipids and its presence in the total neutral sphingolipid fraction suggest that LCB metabolism of these different sphingolipid classes involves different pathways and/or cellular compartments. In the present study, the neutral sphingolipid class(es) with C_{20} sphingosine was not identified. The incorporation of stearate into the LCB of individual components in neutral sphingolipid fraction should be examined to determine whether it is incorporated into C_{20} sphingosine, as demonstrated in other cell types (41).

Cilia

It is now well established that the ciliary membrane of *P. tetraurelia* is highly enriched in ethanolamine sphingophospho- and sphingophosphonolipids (22–27). In the present study, we found that cilia of *P. tetraurelia* also contain a GSL. Although PPnE and PPsE are dominant components among the ethanolamine sphingolipids, phytosphingosine was not detected in the neutral sphingolipid fraction containing the GSL. Thus, the putative enzymes responsible for the conversion of DPsE and DPnE to their phytosphingosine analogs, PPsE and PPnE, may not act on glycosylated sphingolipid substrates. Alternatively, the GSL is expected to occur on the outer leaflet of the cell surface ciliary membrane, and thus this GSL may be physically isolated from the putative enzymes required for in situ metabolism of its LCB moiety.

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